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Journal of NEUROCHEMISTRY

VOLUME 2, 1957/1958

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Pergamon Press

London • New York *Paris • Los Angeles*

JOURNAL OF NEUROCHEMISTRY

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Publishing Offices: 4 & 5 *Fitzroy Square, London W.*1 (*EUS* 4455)

Issued in parts. Subscription per volume (*A*) £6 (*U.S.A.* $17.00) *including postage;* (*B*) *for individual subscribers certifying that the journal is for their private use,* £3 10s. (*U.S.A.* $10.00) *including postage. Payment must be made in advance.*

Distributed by I. R. Maxwell & Co. Ltd., 4 & 5 *Fitzroy Square, London W.*1 *and Pergamon Press, Inc.,* 122 *East* 55*th Street, New York* 22, *N.Y.*

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Journal of Neurochemistry, 1957, Vol. 2, pp. 1–10, Pergamon Press Ltd., London

THE METABOLISM OF INDIVIDUAL PHOSPHOLIPIDS IN THE RAT BRAIN DURING HYPOGLYCAEMIA, ANAESTHESIA AND CONVULSIONS

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(*Received* 15 *May* 1957)

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LITTLE is yet known about the function of the large amount of phospholipid present in cerebral tissue. For many years it was considered to have a purely structural function, but recent experiments on the cat brain perfused *in situ* with glucose-free media have suggested that, under these conditions, phospholipids can serve as a source of energy for cerebral function (ABOOD and GEIGER, 1955). It has also been suggested that certain phospholipids may play a part in the transport of ions within the nervous system (SLOANE-STANLEY, 1952; FOLCH, LEES, and SLOANE-STANLEY, 1957).

DAWSON and RICHTER (1950) first showed that the brain phospholipids as a whole are metabolically active *in vivo*. Using radioactive phosphorus they found that the rate of turnover of phospholipids in the mouse brain is equivalent to that required for complete replacement every 70 hr. They also demonstrated changes in the rate of synthesis under different experimental conditions. No work has hitherto been carried out on the rate of turnover *in vivo* of individual brain phospholipids however†, though DAWSON (1955) has given values for the rates in the liver.

In this paper, studies on the uptake of radioactive phosphate into individual brain phospholipids are described together with the effects of anaesthesia, insulin hypoglycaemia and electrically induced convulsions. The effect of chlorpromazine sedation on the phospholipid metabolism has been discussed elsewhere (ANSELL and DOHMEN, 1956).

EXPERIMENTAL

Young albino rats, four to six weeks old and of 40–60 g body weight, of a strain bred for many years in the department, were consistently used. They were injected intraperitoneally with 3 mc ^{32}P-orthophosphate/kg body weight. After an exchange period of 3 hr, the animals were decapitated and the brains quickly removed. Trichloroacetic acid (TCA) extracts of this tissue were prepared and the lipids extracted from the TCA-insoluble fraction with acetone and chloroform : methanol as previously described (ANSELL and DOHMEN, 1956). Samples of the lipid extract, equivalent to about 300 mg brain tissue, were taken for the measurement of the specific radioactivity of individual phospholipids by the method of DAWSON (1954).

Duplicate samples were oxidized with perchloric acid and the ^{32}P determined by counting in solution as inorganic orthophosphate in a liquid counter. Phosphate estimations were routinely carried out by the method of FISKE and SUBBAROW (1925), but, where the amounts were small, the method of BERENBLUM and CHAIN (1938) was used.

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† *Note added in proof.* Some experiments in which the uptake of intracisternally injected ^{32}P into certain cat brain phospholipids was measured after a long exchange period have recently been cited by ROSSITER, R. J. (1957) in *The Metabolism of the Nervous System* p. 230. (Edited by D. RICHTER) Pergamon Press, London.

Anaesthesia

Anaesthesia was induced by the intraperitoneal injection of thiopentone ('Pentothal' Abbott), 60 mg/kg body weight, 20 min before the injection of ^{32}P, and the animals were kept in a warm place, so that the body temperature was maintained.

Insulin hypoglycaemia

The rats were deprived of food for 16 hr before the experiment. Insulin (80 units/kg body weight) was injected intraperitoneally, 15 min before the injection of the labelled phosphate, and the rats were killed after an isotopic exchange period of 3 hr. Convulsions set in approximately 2 hr after the injection of insulin, and when the animals were killed they were in hypoglycaemic coma.

Electrically induced convulsions

Convulsions were induced with square waves (200 v, 50 c/s, pulse width 10 msec) applied to the shaved scalp of the animal just posterior to the eyes by steel electrodes. The stimulation was carried out for 10 sec when convulsions of 20–80 sec duration invariably set in.

RESULTS

Phospholipid metabolism in the normal rat brain

In Figs. 1 and 2 the uptake of ^{32}P from the acid-soluble fraction into the individual phospholipid fractions over a period of 7 hr is shown. The specific activity ratio (ratio of the specific radioactivity of the individual phospholipid P to that of the acid-soluble P) after a 3 hr exchange period was used as a measure of synthesis (DAWSON and RICHTER, 1950). In Table 1 are given the ratios for total phospholipid, diphosphoinositide, phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl serine and an 'unknown' phospholipid. The rate of uptake of phosphorus into total rat brain phospholipid was similar to that obtained for mouse brain by DAWSON and RICHTER (1950).

It can be seen that the rates of uptake into the individual phospholipid fractions show considerable variation. Of the individual phospholipids studied the greatest relative incorporation in the 3 hr period occurred with the diphosphoinositide. Studies *in vitro* have shown that this phospholipid has an uptake of labelled phosphate second only to that of phosphatidic acid but free phosphatidic acids have not been detected in tissues *in vivo* (DAWSON, 1954; ROUSER, MARINETTI, WITTER, BERRY and STOTZ, 1956) and it is likely that they are rapidly dephosphorylated to yield α,β-diglycerides as soon as they are formed (KENNEDY and WEISS, 1956).

The uptake of ^{32}P into phosphatidyl choline was greater than that into phosphatidyl ethanolamine; it is likely that the radioactivity in the glycerylphosphoryl-ethanolamine spot separated by paper chromatography derives in part from the

TABLE 1.—UPTAKE OF ^{32}P INTO BRAIN PHOSPHOLIPIDS *in vivo* AFTER A 3 HR EXCHANGE PERIOD

	Number of rats	$\frac{\text{Specific radioactivity phospholipid P}}{\text{Specific radioactivity acid-soluble P}} \times 1000$	S.D.
Diphosphoinositide	12	558	±104
'Unknown'	8	136	± 20
Phosphatidyl choline	15	53·5	± 5·0
Phosphatidyl ethanolamine	15	29·1	± 4·9
Phosphatidyl serine (whole brain)	—	<8·0	—
Phosphatidyl serine (cortex)	3	<8·7	—
Phosphatidyl serine (white matter)	4	<6·4	—

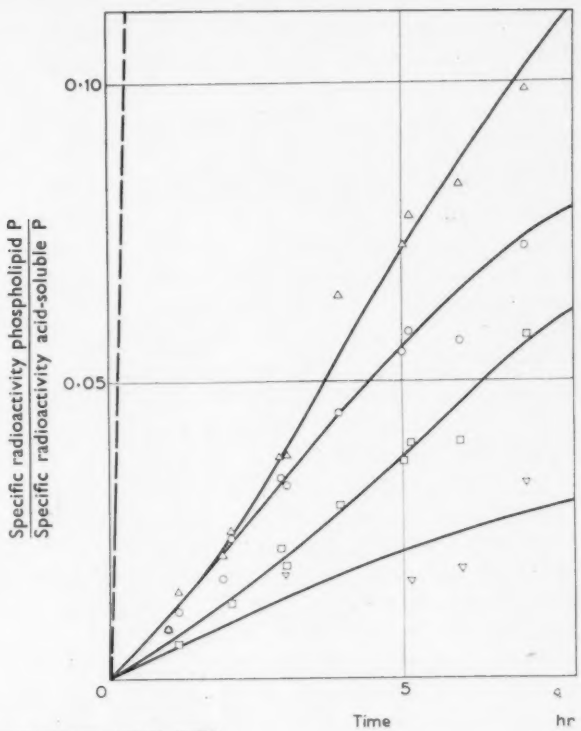

FIG. 1.—The uptake of ^{32}P into the phospholipids of rat brain *in vivo*.
○ Total phospholipid
△ Phosphatidyl choline
□ Phosphatidyl ethanolamine
▽ Phosphatidyl serine
— — Diphosphoinositide

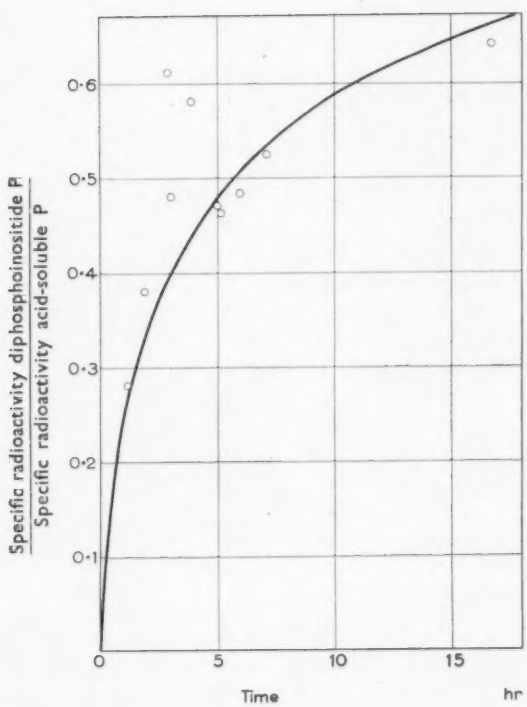

FIG. 2.—The uptake of ^{32}P into rat brain diphosphoinositide *in vivo*.

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ethanolamine-containing acetalphospholipid (ANSELL and DOHMEN, 1956). Phosphatidyl serine had the lowest uptake of the phospholipids identified in brain tissue; the radioactivity present in the glycerylphosphorylserine spot separated by paper chromatography was often not significantly higher than the background. It was considered possible that the activity of the phosphatidyl serine might be lower in the white matter, but the experiments gave no evidence that this was so (Table 1).

'Unknown' phospholipids

The specific radioactivity of the 'unknown' phospholipid was determined from the radioactivity in a water-soluble phosphorus-containing hydrolysis product which moved to a position similar to that of glycerophosphate in the phenol/NH_3 and *tert.*-butanol/TCA solvents used for chromatography. It appeared possible that this lipid might be similar to an unidentified choline-containing phospholipid present in blood serum and shown by DOUSTE-BLAZY (1955) to have a high rate of ^{32}P uptake. If such a phospholipid is present in brain tissue then the water-soluble product of its mild alkaline hydrolysis might be expected to contain choline. For the following reasons however choline was considered to be absent.

(a) When a chromatogram was examined for choline with the phosphomolybdic acid/stannous chloride technique of LEVINE and CHARGAFF (1951), none could be detected in the spot. Esterified choline (e.g. as in phosphorylcholine and glycerylphosphorylcholine) reacts under these conditions (HUENNEKENS, HANAHAN and UZIEL, 1954).

(b) In further experiments the hydrolysis product of the unknown lipid was located on a number of chromatograms by the method of WADE and MORGAN (1953) and eluted from the paper with water. The eluate was then heated at 120° for 24 hr in 2 N-hydrochloric acid and the product of prolonged acid hydrolysis examined for free choline by (i) paper chromatography in *n*-butanol/diethylene glycol/water (4:1:1) (LEVINE and CHARGAFF, 1951), (ii) precipitation with ammonium reineckate; for this an amount of hydrolysate equivalent to 22 μg P was used. No choline could be detected.

The phosphorus of the original alkaline hydrolysis product was labile to periodic acid at room temperature; this determination was carried out by the method of ANSELL and NORMAN (1956). After prolonged acid hydrolysis as described above, only about half the phosphorus originally present was in the inorganic form. This evidence suggests that the original alkaline hydrolysis product may in fact be glycerophosphate; if this is so then, by virtue of its high specific radioactivity, it must have derived from a phospholipid other than phosphatidyl choline, phosphatidyl ethanolamine or phosphatidyl serine.

Two additional water-soluble phosphorus compounds deriving from brain lipids were obtained on the chromatograms and these spots were probably identical with those obtained from liver lipids by DAWSON (1954); they had a very low ^{32}P content and the lipids from which these spots derived must have contributed very little to the total radioactivity of the brain lipids.

Phospholipid metabolism in thiopentone anaesthesia

During thiopentone anaesthesia of three hours duration, there was a general decrease in the uptake of ^{32}P into the phospholipids in agreement with the observation of DAWSON and RICHTER (1950). The incorporation of ^{32}P into phosphatidyl choline

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TABLE 2.—THE EFFECT OF THIOPENTONE ANAESTHESIA ON THE UPTAKE OF ^{32}P INTO RAT BRAIN PHOSPHOLIPIDS AFTER A 3 HR EXCHANGE PERIOD

	Number of rats	Specific radioactivity ratios × 1000					
		Phospholipid P / Acid-soluble P	S.D.	Phosphatidyl choline P / Acid-soluble P	S.D.	Phosphatidyl ethanolamine P / Acid-soluble P	S.D.
Normal	3	48·3	±1·0	59·3	±2·6	37·2	±0·4
Anaesthesia	6	14·9	±3·4	15·1	±6·0	12·1	±2·8
P		<0·001		<0·001		<0·001	

was reduced to 25 per cent and into phosphatidyl ethanolamine to 33 per cent of that of the control animals (Table 2). In these experiments the phosphatidyl serine synthesis was difficult to measure because of the low uptake into the lipid in the normal animal. The experiments also indicated a reduction of diphosphoinositide synthesis to <20 per cent whilst that of the 'unknown' phospholipid was reduced to about 60 per cent.

It was observed that the specific radioactivity of the acid-soluble fraction as a whole was unchanged by thiopentone anaesthesia. However, as the individual specific radioactivities of inorganic phosphate and the energy-rich phosphate esters were not measured, it is not known whether the primary effect of the thiopentone anaesthesia is the inhibition of the formation of these phosphate esters or inhibition of a later step in phospholipid synthesis.

Phospholipid metabolism in insulin hypoglycaemia

Insulin hypoglycaemia caused a considerable decrease in the uptake of ^{32}P into all the phospholipid fractions (Table 3). The percentage decreases, however, were not as great as with thiopentone anaesthesia; thus phosphatidyl choline synthesis and phosphatidyl ethanolamine synthesis were decreased to 60 per cent and 72 per cent respectively of that of the control animals. (The difference in the figures obtained for the percentage decrease in ^{32}P uptake in these two phospholipids was hardly

TABLE 3.—THE EFFECT OF INSULIN HYPOGLYCAEMIA ON THE UPTAKE OF ^{32}P INTO RAT BRAIN PHOSPHOLIPIDS AFTER A 3 HR EXCHANGE PERIOD

	No. of rats	Specific activity ratios × 1000									
		Phospholipid P / Acid-soluble P	S.D.	Phosphatidyl-choline P / Acid-soluble P	S.D.	Phosphatidyl-ethanolamine P / Acid-soluble P	S.D.	Diphospho-inositide P / Acid-solube P	S.D.	Phosphatidyl-serine P / Acid-soluble P	S.D.
Normal	3	32·7	±1·8	38·9	±1·7	21·6	±1·7	402	±40	10·5	±1·9
Insulin hypoglycaemia	3	22·0	±1·1	23·4	±2·0	15·5	±0·2	282	±39	8·0	
P		<0·01		<0·01		<0·05		<0·05		—	

The six animals used were litter mates.

significant ($P = <0{\cdot}1$). The synthesis of phosphatidyl choline and of phosphatidyl ethanolamine were reduced in similar proportion to that observed in anaesthesia.

Phospholipid turnover in electrically-induced convulsions

No significant change in the uptake of ^{32}P into the phospholipids was observed, when stimulation was carried out during the isotopic exchange period. This was true whether stimulation was carried out early in the exchange period, intermittently, or just prior to death. Neither could any significant change be detected if rats frozen in liquid oxygen during convulsions were compared with control animals similarly frozen. A typical group of experiments is recorded in Table 4 in which litter mates were used; the animals were divided into pairs of one control animal and one convulsed animal. No change was observed in the specific radioactivity of the acid-soluble phosphate.

In Table 4 are also collected the mean values of the specific activity ratios obtained in a series of experiments, in which animals were stimulated at various points during the exchange period. There would appear to be a slight increase in the uptake of ^{32}P into phosphatidyl choline but this is not statistically significant ($P = 0{\cdot}2$–$0{\cdot}1$). For comparative purposes, convulsions were induced in one group of animals by the intraperitoneal injection of picrotoxin (12 mg/kg body weight), but no effect on the uptake of ^{32}P into the phospholipids could be detected (Table 5).

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DISCUSSION

The results show that the phosphate moieties of individual brain phospholipids have very different rates of replacement. It is apparent that the uptake of ^{32}P-labelled phosphate into diphosphoinositide proceeds at a rate nearly ten times that of phosphatidyl choline and twenty times that of phosphatidyl ethanolamine. The incorporation of ^{32}P into phosphatidyl serine appears to be much slower than that into the other three phospholipids investigated. It should be borne in mind however that in the present investigation only the replacement of the phosphate moiety of the phospholipid molecule was measured. The rate of replacement of other parts of the molecule may be greater or less. It is known, for example, that [1-^{14}C]glycerol is incorporated *in vitro* more or less uniformly into phospholipid fractions whilst the incorporation of ^{32}P-labelled phosphate is variable (HOKIN and HOKIN, 1955).

It is of interest that the high rate of diphosphoinositide synthesis is apparently peculiar to brain tissue because, as found by DAWSON (1955) in liver tissue, the uptake of labelled phosphate into phosphatidyl ethanolamine and phosphatidyl choline is greater than that into the inositol-containing phospholipid (in liver probably a monophosphoinositide (MCKIBBIN, 1956). The fact that the liver is continuously synthesizing plasma phospholipids may possibly account for the higher rate of synthesis of ethanolamine and choline-containing phospholipids in this tissue. No special function has been definitely ascribed to brain diphosphoinositide, though it has been suggested that it may play an active part in cation transport within the tissue (SLOANE-STANLEY, 1952; FOLCH, LEES and SLOANE-STANLEY, 1957).

The very slow rate of phosphatidyl serine synthesis is also of interest and suggests that this lipid may play a structural role. Recently ROBINS, EYDT and SMITH (1956) have found that kephalins are as much a constituent of myelin as cerebrosides, cholesterol, and sphingomyelins; these kephalins are predominantly phosphatidyl serines (BRANTE, 1949).

TABLE 4.—THE EFFECT OF ELECTRICALLY INDUCED CONVULSIONS ON THE UPTAKE OF ^{32}P INTO RAT BRAIN PHOSPHOLIPIDS

Expt. No.	Treatment	Number* of rats	Mean weight (g)	Specific radioactivity ratios × 1000					
				Phospholipid P / Acid-soluble P		Phosphatidyl choline P / Acid-soluble P		Phosphatidyl ethanolamine P / Acid-soluble P	
				Control	Convulsed	Control	Convulsed	Control	Convulsed
1	Two stimulations during a 5 hr exchange period, animals frozen whole whilst in convulsions	6	55	41·7 ± 3·1	47·0 ± 2·2	61·0 ± 1·4	64·8 ± 3·9	27·3 ± 1·3	32·2 ± 2·4
2	Five stimulations during a 3 hr exchange period	6	51	43·9 ± 2·7	44·0 ± 1·8	56·9 ± 4·6	58·8 ± 2·3	29·7 ± 0·7	28·2 ± 1·3
3	Animals under thiopentone anaesthesia. Four stimulations during a 3 hr exchange period	6	60	17·0	14·9	18·0	16·0	12·0	8·9
4	Stimulations at various points during a 3 hr exchange period	30	about 50	39·6 ± 4·8	39·7 ± 4·3	46·9 ± 7·7†	52·3 ± 6·5‡	28·4 ± 3·1†	28·3 ± 4·9‡

* Half were control animals
† Mean of 12 animals
‡ Mean of 11 animals

Values are given ± S.D.

Groups of litter mates were used in experiments 1, 2, and 3. Experiment 4 includes data from experiment 2.

TABLE 5.—THE EFFECT OF PICROTOXIN CONVULSIONS ON THE UPTAKE OF ^{32}P INTO BRAIN PHOSPHOLIPIDS AFTER A 3 HR EXCHANGE PERIOD

	No. of rats	Specific radioactivity ratios × 1000							
		Phospholipid P / Acid-soluble P	S.D.	Phosphatidyl choline P / Acid-soluble P	S.D.	Phosphatidyl ethanolamine P / Acid-soluble P	S.D.	Diphosphoinositide P / Acid-soluble P	S.D.
Normal	3	38·9	±3·0	44·5	±2·8	24·2	±2·6	552	±3
Picrotoxin† convulsions	3	35·7	±2·1	44·7	±4·5	22·8	±3·0	471	±101

† Picrotoxin (12 mg/kg body weight) was injected 30 min before decapitation of the animals. They were in violent convulsions. All six animals were litter mates.

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The depressant effect of thiopentone anaesthesia was very marked and was greater than that obtained with chlorpromazine (ANSELL and DOHMEN, 1956). This effect is likely to be a reflection of the capacity of barbiturates to uncouple oxidative phosphorylation in nervous tissue *in vitro* (BRODY and BAIN, 1951, 1954). It may well be that anaesthetics in general have this property, since diethyl ether has a similar effect on brain mitochondrial preparations (HULME and KRANTZ, 1955); on the other hand, xenon and nitrous oxide, at anaesthetic concentrations, do not have this action (LEVY and FEATHERSTONE, 1954). It is possible, however, that anaesthetics inhibit some of the more specific reactions necessary for phospholipid synthesis e.g. the reaction between cytidine triphosphate and phosphoryl choline which is an essential step in the biosynthesis of phosphatidyl choline (KENNEDY and WEISS, 1956).

Effects obtained with preparations *in vitro* cannot however, necessarily be equated with those obtained in experiments *in vivo*. Low concentrations of chlorpromazine (0·1 mM equivalent to about 32 mg/kg body weight) increase the uptake of labelled phosphate into diphosphoinositide and phosphatidyl serine and decrease the uptake into phosphatidyl ethanolamine and phosphatidyl choline *in vitro*; higher concentrations (1 mM equivalent to about 320 mg/kg body weight) cause a general decrease (MAGEE, BERRY and ROSSITER, 1956). Further experiments (ANSELL, 1957) have shown that low doses of chlorpromazine *in vivo* cause only an inhibition of synthesis though this is less marked than with the higher doses used (ANSELL and DOHMEN, 1956).

The changes observed in insulin hypoglycaemia confirm those obtained by DAWSON and RICHTER (1950) and show that there is a general reduction in the uptake of labelled phosphate into the four phospholipids investigated. In liver tissue insulin accelerates the breakdown of phosphatidyl choline with the concomitant production of glycerylphosphorylcholine (DAWSON, 1956): in this tissue the breakdown probably reflects the mobilization of the lipid fatty acids in hypoglycaemia conditions.

It is apparent from the results obtained on animals subjected to convulsions that any change that might occur in the rate of phospholipid metabolism was not measured by the methods used. No significant change was found in the uptake of labelled phosphate into the brain phospholipids, whether stimulation was carried out early in the exchange period, continuously or at the end. Neither did freezing the convulsing animals reveal any change which might be masked by post mortem artefacts.

DAWSON and RICHTER (1950) found that convulsions caused a slight fall (about 10 per cent) in the rate of synthesis of mouse brain phospholipids and TORDA (1954) has also reported that convulsions induced under different conditions reduced the radioactivity present in rat brain phospholipids by 17 per cent. These changes are comparatively small and indicate that if there is any major change in phospholipid metabolism during convulsions, the phosphate moiety is but little involved. It is possible however that other parts of the phospholipid molecule are affected by electrical stimulation. GEIGER, YAMASAKI and LYONS (1956) have shown that stimulation of the brain cortex of cats *via* the brachial plexus causes a decrease in the total lipid nitrogen and an increase in the amino nitrogen of the lipids of the brain.

SUMMARY

(1) The uptake of ^{32}P into the individual phospholipids of rat brain *in vivo* has been studied and considerable variation in turnover was observed. The rate of uptake

decreased in the order: diphosphoinositide > phosphatidyl choline > phosphatidyl ethanolamine > phosphatidyl serine.

(2) In thiopentone anaesthesia the incorporation of ^{32}P into phosphatidyl choline was reduced to 25 per cent and into phosphatidyl ethanolamine to 33 per cent of the normal. In insulin hypoglycaemia the decreases were to 40 per cent and 72 per cent respectively.

(3) No significant effect on the incorporation of ^{32}P into rat brain phospholipids was observed as a result of electrically induced convulsions.

Acknowledgements—The authors thank Dr. DEREK RICHTER for his advice and encouragement throughout and Dr. R. M. C. DAWSON for helpful suggestions. One of us (H. D.) is indebted to the Rockefeller Foundation for a personal grant, and we thank the Medical Research Council for a grant in aid of expenses. The skilled assistance of Miss ANNE PHILLIPS is gratefully acknowledged.

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PREPARATION OF TISSUE SLICES FOR METABOLIC STUDIES: A HAND-MICROTOME ESPECIALLY SUITABLE FOR BRAIN

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(*Received* 4 *April* 1957)

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THE simple apparatus described herein was designed primarily as an answer to the difficulties encountered in preparing slices of fresh rat brain for metabolic studies *in vitro*. It proved subsequently to be an efficient tissue slicer for general use.

In connection with a study of lipide biosynthesis by nervous tissue *in vitro* (MAJNO and KARNOVSKY, 1957) it became necessary to prepare standard tangential slices of rat cerebral cortex. Many workers in this field have used the Stadie-Riggs microtome

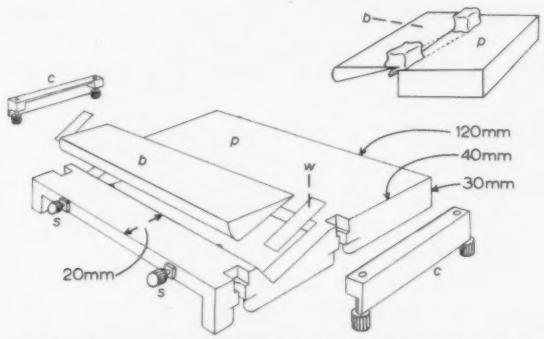

FIG. 1.—Exploded view of the apparatus, and (inset) schematic representation of its working principle. Further explanations in text.

(STADIE and RIGGS, 1944). This apparatus yields excellent results with various tissues, but it is not entirely successful when applied to brain. Many slices begin to form satisfactorily, then disintegrate as the blade advances. This occurs primarily because the slice remains trapped between the moving blade and a motionless surface until the cut is completed. The design of the slicer described here aims at eliminating this feature: i.e. the slice becomes free as fast as it is being cut.

The principle is shown in Fig. 1 (inset). A blade *b* is mounted along the edge of a horizontal plate *p*. The cutting edge is slightly raised above the edge of the plate. The tissue is placed at one end of the blade, then advanced along the cutting edge. The slice appears free on the under-side of the blade.

CHARACTERISTICS OF THE APPARATUS

Fig. 1 shows an exploded view of the apparatus, which is built of lucite, 8 mm thick for the body and 2 mm thick for the clamps *c*. Overall dimensions are not critical, except for the length (12 cm) which is determined by the length of the knife. This is a standard 120 mm microtome blade. If placed on the supports without washers, and brought exactly edge to edge with the lucite plate *p* by

manipulating the screws *s*, its upper surface should be flush with that of the plate. It is raised to the desired height—which determines the thickness of the slice—by means of two rectangular washers of known thickness *w*. A simple way of preparing such washers is to use standard aluminium foil for domestic use (Reynolds Wrap, thickness approx. 15 μ), repeatedly folded, pressed, trimmed and checked with a caliper. These flexible washers proved more satisfactory than stiff metal strips cut from calibrated sheets.

USE OF THE APPARATUS

For assembling the slicer. The washers are first placed on the supports, then the blade is rested on the washers and adjusted with the screws *s* in such a way that the cutting edge barely overlaps the plate. An overlap of a fraction of 1 mm is sufficient. The clamps are applied and tightened.

For cutting tissue other than brain. (1) The tissue is trimmed to a block of the proper size, approx. 15 × 15 × 8 mm; (2) The surface of the plate and *both* sides of the blade are moistened with an appropriate physiological solution; (3) The apparatus is placed on a table with the cutting edge towards the right; (4) The block is placed against the edge of the blade, as indicated in Fig. 1 (inset); (5) The tissue is covered with a small rectangle of *dry* filter paper (Fig. 2-A). The paper adheres firmly to the tissue and facilitates further manipulations. (6) With very gentle pressure the tissue is moved along the blade in such a manner that at the end of a single stroke the block will be entirely on the upper surface of the blade (Fig. 2-A). (7) The instrument is turned upside down. The slice, which adheres to the blade, should be scooped up with the tips of a pair of fine forceps or better with a fine paintbrush wetted with physiological solution. (8) At frequent intervals both faces of the blade should be wiped clean with cleansing tissue moistened with saline.

For cutting rat brain. A single hemisphere is used, covered with filter paper, convex side down. A finger is placed on the interhemispheric surface. One stroke will shave approximately 2/3 of the lateral surface. Fig. 2-B shows six such slices, both 'first' and 'second', obtained from 3 hemispheres of weanling rats (washers 0·44 mm). If more tissue is desired the block can then be rotated by 90 degrees —hypothalamus facing upwards—and a smaller slice is shaved off the dorsal surface. The total weight of the two slices, for the hemisphere of a 250 g rat, is 30–60 mg, depending on the thickness of the washers. Deeper slices are easily obtained; their metabolic characteristics are not identical with those of the superficial slices (MAJNO and KARNOVSKY, 1957).

The slicing should proceed without difficulty. If the slices tend to disintegrate, either a dull blade, or the presence of tissue debris beneath the blade are usually responsible. Debris can be removed without dismantling the apparatus.

Wetting of all surfaces, though not absolutely necessary, greatly facilitates the procedure. With firm tissues, such as liver or kidney, several slices can be cut in sequence and collected together.

After use the blade should be thoroughly dried and protected with vaseline; discoloured areas can be removed with fine abrasive powder. If the tissue sliced is brain, the cutting edge lasts for a long time without requiring additional care. In slicing rat liver it was found advisable to strop, and occasionally to hone, the blade between experiments. Old knives are sometimes greatly deformed by honing, in that the bevel angle becomes excessively obtuse. Such knives should not be used with the slicer unless the edge is restored to the proper shape.

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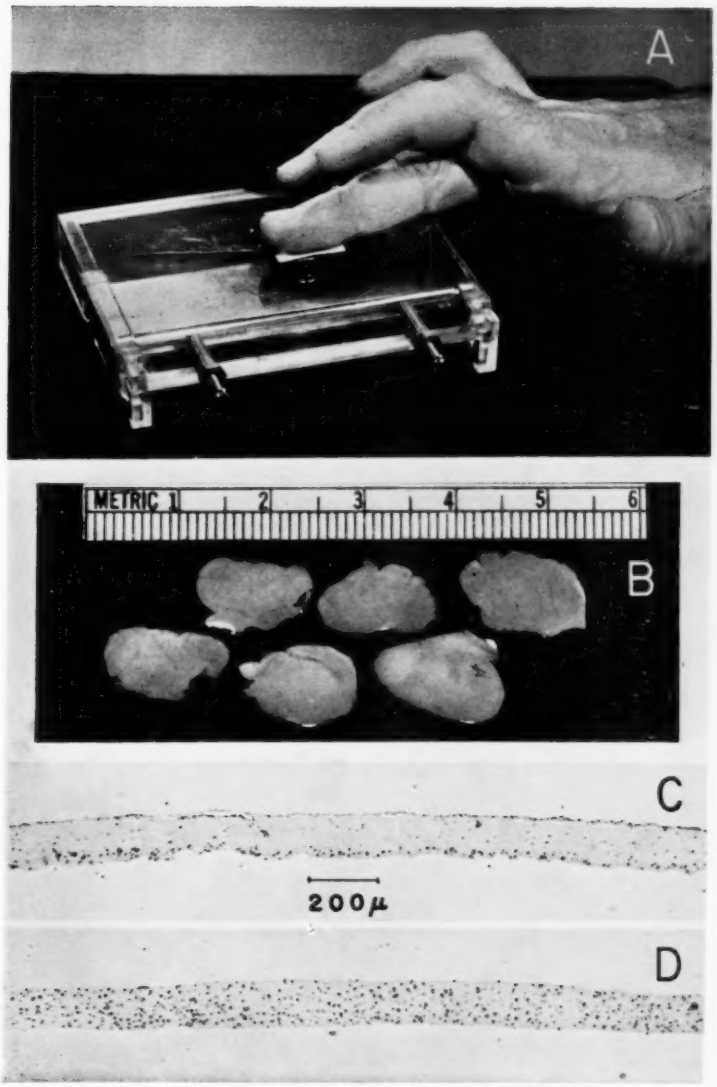

FIG. 2.
(A): The apparatus, shown in the process of slicing rat liver.
(B): First and second slices from 3 hemispheres of rat brain, photographed in saline.
(C) and (D): Histological aspect of a first (C) and of a second (D) slice of rat brain. Further explanations in text.

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RESULTS AND DISCUSSION

In order to test the accuracy of the apparatus, 40 slices were cut in sequence from the brains of 10 weanling rats, using 0·33 mm washers for the first 20 slices and 0·44 mm washers for the remainder. A first and a second slice were cut from each hemisphere; each slice was weighed to the nearest 0·2 mg, then placed in saline and taken up on millimetre squared paper. Assuming that the tissue has a specific gravity of unity, the average thickness can be calculated by dividing the weight in mg by the number of mm^2 covered by the tissue. The area covered by the first slices could be determined quite accurately. The second slices, lacking the support of a pial membrane, were liable to overstretch slightly on the millimetre paper; for this reason they appeared to be 4–8 per cent thinner, and tended to be more variable, than the first slices (Table 1).

TABLE 1.—WEIGHT AND THICKNESS OF FIRST AND SECOND SLICES PREPARED FROM THE CEREBRAL CORTEX OF WEANLING RATS

	I slices	II slices	I slices	II slices
Thickness of washers (mm)	0·44	0·44	0·33	0·33
Number of slices	10	10	10	10
Average weight of slices (mg)	26·8	30·2	13·9	15·4
Average thickness (mm)	0·38	0·35	0·23	0·22
± standard error	0·021	0·015	0·016	0·027

In all series the slices were 70–100 micra thinner than the corresponding washers; the standard deviation for single samples was ±5·5 per cent and ±4·3 per cent for the thicker series, and ±6·1 and ±12·3 for the thinner.

Another set of slices was fixed in formalin, embedded in paraffin, cut transversally and stained (Nissl). Fig. 2 shows representative samples of a first (C) and of a second (D) slice cut with 0·33 mm washers. Each slice appears to be thinner than the expected 0·23–0·22 mm. This is accounted for by the 45 per cent linear shrinkage which occurs in brain tissue during the embedding procedure (ROBINS, SMITH and EYDT, 1956). The thickness was satisfactorily uniform throughout any given cross-section. With the thinner setting the first slice corresponds very closely to the cell-scarce plexiform layer. This is less than half the maximum thickness compatible with adequate diffusion of oxygen throughout slices of rat cerebral cortex in media equilibrated with pure O_2 (FIELD, 1948),

The sections showed practically no evidence of trauma to the tissue. It is true that mechanical trauma, such as 'smearing', has little or no effect on the oxygen uptake of brain slices (ELLIOTT and HENDERSON, 1948); however, it does depress lipide biosynthesis *in vitro*, and should be avoided (MAJNO and KARNOVSKY, 1957).

Frontal sections of cat brain were prepared in order to verify whether the white matter (which is very scanty in rat brain) would lend itself to slicing. The preparations were excellent; it may be noted, however, that for studies on white matter *in vitro* other techniques are available which do not require slicing (MAJNO and KARNOVSKY, 1957).

Lately, MCILWAIN *et al.* prepared slices with an apparatus of special design

operating on the principle of a mechanically-driven blade which descends on the tissue with a quick 'chopping' action (McILWAIN and BUDDLE, 1953). There is probably still a need for a less sophisticated apparatus, of much simpler construction, and possibly better suited when a 'shaving' action is desired, such as in studies of cerebral cortex.

The apparatus described has been very satisfactory with respect to reproducibility and speed of operation. Waste of tissue was reduced to a minimum, and the adjustable thickness was found to be very advantageous. These characteristics might make its use appropriate when large amounts of slices are needed for metabolic studies.

SUMMARY

Details are given for the construction of a simple tissue slicer, consisting of a microtome blade mounted along the edge of a lucite plate. The apparatus is particularly useful in preparing tangential slices of desired thickness from cerebral cortex. For superficial slices of rat brain, cut at 0·38 and 0·23 mm, the standard error was ± 21 and ± 16 μ respectively.

Acknowledgements—This work was supported wholly by Grant No. 75-2 from the National Multiple Sclerosis Society. It was carried out during the tenure by one of us (G. M.) of a Lederle Medical Faculty Award. We are indebted to Dr. MANFRED L. KARNOVSKY for his most helpful advice, and to Mrs. CLAUDIA FROESCH and Mrs. MONIKA LA GATTUTA for their skilful assistance.

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Journal of Neurochemistry, 1957, Vol. 2, pp. 15-20, Pergamon Press Ltd., London

THE PYRIDINE NUCLEOTIDE AND DIPHOSPHOPYRIDINE NUCLEOTIDASE LEVELS OF THE BRAIN OF YOUNG RATS

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(*Received* 4 *April* 1957)

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IN COMMON with most mammals, the brain tissue of the young rat undergoes marked changes in composition during the period from birth until the myelination process has been largely completed. Since the pyridine nucleotides are of importance to the metabolism of brain tissue, the total pyridine nucleotide content of the brain was studied as a function of age. An enzyme catalysing the hydrolysis of DPN (diphosphopyridine nucleotide) at the nicotinamide-ribosyl bond, DPNase, was studied concurrently. It seemed probable that these studies would also contribute to the elucidation of the questions (1) is DPNase solely a degradative enzyme *in vivo* or is it also associated with synthesis of DPN? and (2) is DPNase, by virtue of its ability to catalyse an exchange with certain pyridine compounds to form analogues of DPN (ZATMAN *et al.*, 1954), partially responsible for the toxicity of pyridine derivatives such as 3-acetyl pyridine?

MATERIALS AND METHODS

The Holzman strain of albino male rats was used in all of the experiments reported. In the experiments, *in vivo*, all of the compounds were administered as aqueous solutions by intraperitoneal injection using concentrations such that each animal received a volume equivalent to 1 per cent of its body weight. Distilled water injections were employed for the control group of rats.

The rats were decapitated, the brains rapidly excised, and the pyridine nucleotides of brain tissue were extracted by the trichloroacetic acid procedure of KAPLAN *et al.* (1954). The oxidized pyridine nucleotides were estimated by the methyl ethyl ketone modifications (CARPENTER and KODICEK, 1950) of the fluorescence procedure of LEVITAS *et al.* (1947). DPNase activity was estimated by the assay system of ZATMAN *et al.* (1953) using the fluorescence procedure to determine DPN concentration before and after incubation. The unit of DPNase activity is that described by ZATMAN *et al.* (1953), i.e., one unit of enzyme activity will catalyse the hydrolysis of 1 μmole of DPN in 60 min at 37°.

The 3-acetyl pyridine employed was freshly distilled before use and obtained from Mr. F. STOLZENBACH. The growth hormone was obtained from Dr. R. W. BATES, National Institutes of Health. Ciba Pharmaceutical Products, Inc. supplied the reserpine (Serpasil).

RESULTS

Pyridine nucleotide content of brain tissue as a function of age. The data plotted in Fig. 1(a) show that from 2 to 10 days following birth, the pyridine nucleotide content of rat brain was relatively constant on a wet weight basis. From the tenth to the twenty-first day, the pyridine nucleotide level increased about 40–50 per cent and then remained constant from 21 days to 8 weeks, the oldest age group studied. When this same data was plotted on a dry TCA weight basis, i.e., dry weight of trichloroacetic acid (TCA) insoluble material (Fig. 1b), the DPN concentration was observed to

start at a high level and decrease to a lower limit occurring at about 21 days of age. The explanation for the differences occurring between the wet and dry TCA weight plots is that the increase in pyridine nucleotide concentration was about 50 per cent,

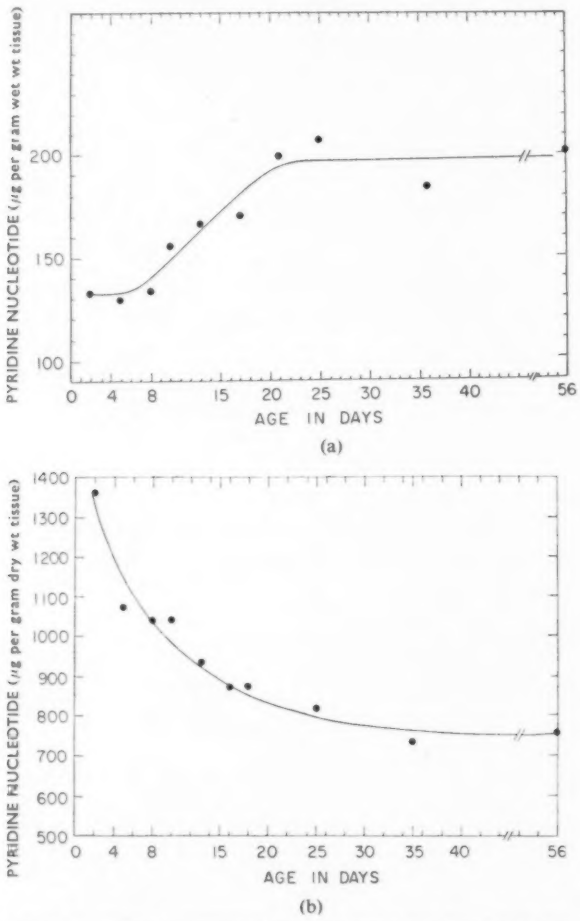

FIG. 1.—*Pyridine nucleotide content of rat brain tissue as a function of age.* Rat brain tissue was obtained and assayed for pyridine nucleotide content as described in the Method section. The data reported represents the average value obtained, at each age, using groups of rats containing a minimum of three animals. The curves were found to be reproducible by repeating the experiment using new groups of rats at the various ages. The wet weight was obtained by weighing the freshly excised brain. The dry weight represents the weight of the solids precipitated by trichloroacetic acid, collected by centrifugation, and dried *in vacuo* over phosphorous pentoxide to constant weight.

whereas the increase in the brain tissue solids insoluble in TCA was approximately 250 per cent (from 9·8 per cent solids to 25 per cent).

DPNase content of brain tissue as a function of age. The data presented in Fig. 2 show that the DPNase level of brain was low at 2 days of age, but increased rapidly about tenfold calculated on a wet weight basis and about fivefold computed on a dry TCA weight basis.

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Distribution of DPNase among particles from brain tissue. It may be observed from the data tabulated in Table 1, that when brain tissue was fractionated by the procedure of BRODY and BAIN (1952), the DPNase activity resided largely (approximately 50 per cent) in the R_4 or microsomal fraction. The nuclear, mitochondrial,

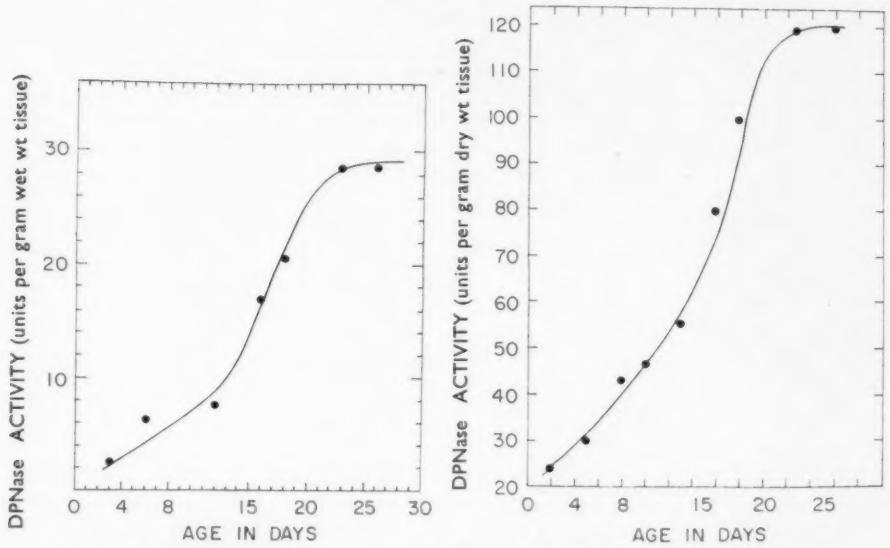

FIG. 2.—*DPNase content of brain tissue as a function of age.* The experimental details have been described in the Method section and in Fig. 1.

TABLE 1.—DISTRIBUTION OF DPNASE IN CELL PARTICULATE FRACTIONS

(Male Holtzman albino strain of rats were decapitated and the brain rapidly excised and placed in ice. After removal of most of the white matter, the tissue from 10 animals was homogenized in cold sucrose solution and fractionated into particulate preparation by the procedure of BRODY and BAIN (1952). Each fraction was washed once with sucrose. DPNase activity was estimated as described in the method section.)

Brain fraction	DPNase activity	
	Expt. 1	Expt. 2
	%	%
R_1 (cell debris and whole cells)	22	23
R_2 (nuclei)	6	9
R_3 (mitochondria)	15	6
R_4 (microsomes)	50	48
R_5 (supernatant liquid)	7	14

and supernatant liquid fractions contained the enzyme in amounts varying from 6–15 per cent of the total activity. The R_1 fraction, containing cellular debris, fragments, and whole cells contained 22–23 per cent of the DPNase activity. These results confirm the observations of JACOBSON and KAPLAN (1957), who found that rabbit brain DPNase was contained in the microsomal fraction.

Nicotinamide induced synthesis of pyridine nucleotides in brain. KAPLAN *et al.* (1954) have demonstrated that the injection of nicotinamide into mice caused a ten-fold increase in the liver pyridine nucleotide level. The pyridine nucleotide in brain also increased, but to a lesser extent. The data presented in Table 2 show that the

TABLE 2.—NICOTINAMIDE-INDUCED SYNTHESIS OF PYRIDINE NUCLEOTIDE

(Male Holtzman albino rats of the appropriate age were given intraperitoneal injections of nicotinamide (50 mg/ml) or distilled water. All injections were 1 per cent of the body weight. After 12 hr, the animals were decapitated, the brains rapidly excised and homogenized in cold 5 per cent TCA, and aliquots were assayed for pyridine nucleotide by the method of KAPLAN *et al.* (1954). The results reported are the average of three rats. The figures in parenthesis refer to the pyridine nucleotide content as μg/g dry weight of TCA-insoluble material.)

	Pyridine nucleotide	
	8-day-old rats	25-day-old rats
	μg/g wet weight	μg/g wet weight
Control (water)	146 (1160)	198 (794)
Nicotinamide (500 mg/kg)	191 (1510)	301 (1200)

administration of nicotinamide produced an increase in the pyridine nucleotide content of rat brain tissue. The pyridine nucleotide increased about 30 per cent in the 8-day-old rat and approximately 51 per cent in the 25-day-old animals. Similar studies have shown that the administration of nicotinamide has no effect on the quantity of DPNase activity present. Other experiments have shown that reserpine, chlorpromazine, and pentobarbital administered *in vivo* had no detectable effect on the *total* pyridine nucleotide or DPNase content of brain tissue. Rats with low DPNase levels (8 days old) were administered growth hormone. These rats failed to show any increase in enzyme synthesis over control animals.

Toxicity of 3-acetyl pyridine. Since it has been proposed that the toxicity of 3-acetyl pyridine might be partially explained by the formation of a 3-acetyl pyridine analogue of DPN catalysed by the exchange reaction of DPNase (KAPLAN *et al.*, 1954) and since KAPLAN and CIOTTI (1954) have evidence for the formation of the analogue in mouse brain following the administration of 3-acetyl pyridine, the toxicity of 3-acetyl pyridine was studied in 8-day-old rats (low DPNase activity in brain tissue) and in 25–30-day-old rats (high DPNase activity). The results of these experiments* indicated that 3-acetyl pyridine was more toxic to the older animal than to the young. The lethal dose (death occurring within 24 hr) was from the range 100–150 mg/kg body weight with the 25-day-old rat to the range 300–600 mg/kg with the 8-day-old animal.

DISCUSSION

It is interesting to note that the pyridine nucleotide content of rat brain was relatively constant on a wet weight basis until the period of active myelination, even though the concentration of proteins and other solids was increasing. Thus, assuming

* The 3-acetyl pyridine was administered as an aqueous solution by intraperitoneal injection. Solutions of the drug in the proper concentrations were prepared so that each animal received a volume by injection equal to 1 per cent of its body weight.

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no specific inter- or intra-cellular distribution,* the pyridine nucleotides would be present at a constant concentration and could satisfy the coenzyme requirement of the developing brain during this early period. When the myelination process accelerates at about the tenth day *post-partum*, the increase in metabolic activity may be such that more pyridine nucleotides could be required and therefore, are synthesized. The data show approximately a 50 per cent increase in the pyridine nucleotide concentration of brain on a wet weight basis.

The increase in DPNase activity was observed to be large until after the most active myelinating period had terminated, i.e., about the 20–22 day *post-partum*. The increase (from 2-day-old rat brain) was close to tenfold on a wet weight basis, but even though a large increase in the brain solids had occurred, a fivefold increase in enzyme action was observed when the data was computed on a dry TCA weight basis. The function of DPNases *in vivo* is not known. The apparent hydrolytic activity of the enzyme is low in the whole cell and becomes apparent only on disrupting the cell.† This observation indicates that DPN is bound and unavailable to the DPNase‡ or that DPNase hydrolytic activity is limited by some control mechanism, or that the DPNase may possess some function *in vivo* other than its *in vitro* hydrolytic action.§ The large difference in the extent of the change in DPNase content when compared to the change in pyridine nucleotide concentration indicates that the DPNase is probably not related to the synthesis of pyridine nucleotides. In addition, this lack of correlation is suggested by experiments which showed that nicotinamide can induce synthesis of pyridine nucleotides in animals with low as well as high brain DPNase activity. The 8-day-old rat showed a nicotinamide induced increase in pyridine nucleotide content of 350 μg per g dry TCA weight, while an increase of 406 μg per g dry TCA weight was observed for the 25-day-old rats. In this same age interval, the DPNase activity increases about threefold on a dry TCA weight basis. It appears that the nicotinamide-induced synthesis of pyridine nucleotides was not related to the quantity of DPNase present.

Woolley (1945) has found that 3-acetyl pyridine causes typical nicotinamide deficiency symptoms, which may be alleviated by increasing the amount of nicotinic acid in the diet. At higher dose levels, 3-acetyl pyridine is toxic and causes the death of mice and rats within 24 hr (Kaplan *et al.*, 1954). It has been postulated by Kaplan *et al.* (1954) that drugs of the pyridine derivative class, e.g. *iso*nicotinic acid hydrazide, marsilid, 3-acetyl pyridine, etc. are toxic due to an exchange catalysed by DPNases.

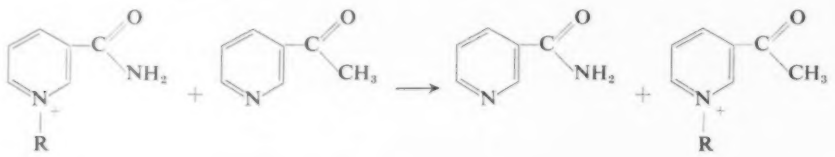
* When brain is fractionated into particles and the pyridine nucleotide content of each fraction determined, the majority of the pyridine nucleotides are found in the supernatant fraction (Jacobson and Kaplan, 1957). This observation does not preclude the possibility that the DPN is bound in the intact living cell.

† McIlwain and Rodnight (1949) observed that DPNase activity is greater in damaged tissue than in carefully excised nervous tissue. They also made a comparison of the velocity of DPN hydrolysis by DPNase with the cellular DPN content and concluded that the cellular DPN would be entirely hydrolysed by the DPNase present, *in vivo*, with a short time interval. A similar comparison made from the data presented in this paper shows that the brain tissue of 8-day-old rats has a ratio of DPN content to DPNase activity of about 1 to 30 (i.e. 0·2 μmole of DPN per g wet weight tissue compared to 6 units of DPNase activity per g wet weight tissue). Thus, in the absence of a mechanism for inhibiting, *in vivo*, DPNase activity and in the absence of DPN synthesis, the DPN content of brain tissue could be entirely hydrolysed in 2 min. A ratio of 1 to 100 may be estimated for DPN to DPNase levels in the 25-day-old rat brain tissues (i.e. 0·3 μmole of DPN per g wet weight of tissue compared to 28 units of DPN activity per g wet weight tissue). This could represent complete hydrolysis of the brain tissue DPN in about 36 sec.

‡ See footnote on previous page.

§ Another possibility, that the DPNase is functioning within the cell as a hydrolytic enzyme with DPN hydrolysis being rapidly replaced by DPN synthesis, could be studied by investigating the turnover-rate of DPN *in vivo*.

Thus, a typical exchange reaction characterized by KAPLAN and CIOTTI (1954) is illustrated by equation (1).



(1)

DPN+ 3-acetyl pyridine Nicotinamide 3-acetyl pyridine analogue of DPN

This type of reaction has been observed both *in vitro* and *in vivo* (KAPLAN *et al.*, 1954; KAPLAN and CIOTTI, 1954). The data presented in this paper show that the lethal dose of 3-acetyl pyridine may be related to the DPNase concentration in brain. It is found that the 8-day-old rat, which had a low level of brain DPNase, was much less sensitive to the pyridine compound than was the 25-day-old rat which had a higher DPNase content. This, of course, does not preclude analogue formation by a synthetic, rather than exchange, mechanism.

SUMMARY

(1) The pyridine nucleotide content of rat brain tissue increased from approximately 130 μg in the 4-day-old animals to about 200 μg per g wet weight tissue in the 20-day-old rat. The pyridine nucleotide level was relatively constant in rats older than 20 days.

(2) The diphosphopyridine nucleotidase (DPNase) activity of rat brain tissues increased from about 2 units in the 2-day-old animal to almost 28 units per g wet weight tissue in the 20-day-old rat. Thereafter, the enzyme level was relatively constant.

(3) The toxicity of 3-acetyl pyridine was correlated with the DPNase activity of the rat brain. Thus, young rats (low DPNase activity) were more resistant to 3-acetyl pyridine toxicity than the older animals (high DPNase activity).

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THE EFFECT OF SOME INORGANIC IONS ON BRAIN SLICES METABOLIZING GLUCOSE OR PYRUVATE

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(*Received* 10 *May* 1957)

ELECTROLYTES play a fundamental role in the metabolism and function of nervous tissue. Several investigations have been made of the functional aspect of the action of potassium and calcium ions (KEYNES and LEWIS, 1955), but little is known about the effect of electrolytes, particularly sodium ions, on the metabolic pattern of brain tissue. Cell metabolism is known to be greatly affected by the structural integrity; thus the metabolism in tissue slices may differ from that in a homogenate of the same tissue. Slices prepared without disrupting the majority of the cells, can presumably reproduce more exactly the state of affairs in the living organism. The effect of some physiologically important electrolytes on the metabolism of brain slices has therefore been studied in the hope of disclosing the relation between functional activity and metabolism in the brain.

CANZANELLI, ROGERS and RAPPORT (1942) investigated the effects of inorganic ions on the oxygen uptake of cerebral cortex slices with special attention to potassium, sodium and calcium. GORE and MCILWAIN (1952) also studied similar problems with special reference to the metabolic response to electrical stimulation. In the present investigation, it was intended to study in further detail the effect of these ions on the metabolism of cerebral tissue with glucose or pyruvic acid as substrates. Not only the oxygen uptake of the brain slices but also substrate utilization and lactic acid formation were measured. A preliminary account of some of these experiments has appeared (TAKAGAKI and TSUKADA, 1957).

METHODS

Preparation of cerebral cortex slices and conditions of incubation. (See also TAKAGAKI, HIRANO and TSUKADA, 1957): Guinea pigs fed on a mixed diet, weighing 200–300 g, were used throughout. The animals were killed by decapitation, and the brain removed undamaged into a small beaker which contained cold incubating solution. Cerebral cortex slices about 0·35 mm thick were cut on a cooled platform with a double-edged safety-razor blade moistened with saline. The slices were collected in incubation medium at 0°.

Pooled slices were weighed on a torsion balance and approximately 120 mg samples were transferred to chilled Warburg flasks containing 3 ml of incubation medium. The Warburg flasks, the gas phase of which was replaced with pure oxygen by the evacuation method, were placed in the thermostat some 30 min after the death of the animal and incubated at 37°. During the incubation period they were shaken in the conventional way and readings of oxygen pressure were taken at 10 min intervals. Usually the incubation period was 90 min including the period of equilibration.

Media. The medium used in all control experiments contained 0·127 M-NaCl, 0·0051 M-KCl 0·00273 M-$CaCl_2$, 0·00127 M-KH_2PO_4, 0·00127 M-$MgSO_4$ and 0·01 M-Na_2HPO_4 buffer of pH 7·0. As the substrate, glucose, sodium pyruvate or lithium lactate was used. In preparing a potassium-free medium, KH_2PO_4 was replaced with NaH_2PO_4, and KCl with NaCl. When potassium ions were increased, KCl was added to the control medium in the desired concentration. In some experiments (Fig. 3) in which KCl was added, the normal tonicity of the medium was maintained by making

the appropriate reduction in NaCl concentration, and 0·05 M-glycylglycine buffer of pH 7·0 was used instead of phosphate buffer. For a calcium-free medium, $CaCl_2$ was omitted from the usual medium, and oxalate was not added. When the sodium-free solution was prepared, choline chloride was substituted for NaCl and K_2HPO_4 for Na_2HPO_4, and the correct osmotic pressure of the medium was maintained. In this case only, potassium pyruvate was used as a substrate. Additional ammonium ions were added as NH_4Cl.

Chemical estimations. At the end of the incubation period, the flasks were removed from the manometers, rapidly chilled in crushed ice, and samples for chemical estimations were pipetted into 5 per cent (w/v) trichloroacetic acid solution. Glucose was estimated by the method of KOEHLER (1952) with the aid of the 'Anthrone' reagent. Pyruvic acid was determined by the method of FRIEDEMANN and HAUGEN (1943) ('procedure B'). This rather simple procedure was believed to be adequate for the present purpose. Lactic acid was determined according to BARKER and SUMMERSON (1941). The utilization of added substrate by respiring cerebral slices was determined by the difference of substrate concentration in the medium before and after the incubation period.

Chemicals. Pyruvic acid (reagent grade, Kanto Chemicals, Tokyo), was neutralized with alcoholic NaOH or KOH after redistillation, and the sodium or potassium pyruvate precipitated according to ROBERTSON (1942). The recrystallized preparation was assayed by the method of LONG (1942); it was found to be practically 100 per cent pure. Anthrone (reagent grade, Kanto Chemicals, Tokyo) was recrystallized from benzene. Sulphuric acid (reagent grade, Mitsubishi Chemical Industries Ltd., Tokyo) was used throughout quite satisfactorily for the determination of lactic acid. Other chemicals were reagent grade and were used without further purification.

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RESULTS

Potassium ions and sodium-free medium. Addition of 100 mM-KCl to the incubation medium causes a pronounced increase in the respiration of cerebral cortex slices with glucose as well as pyruvic acid or lactic acid as substrates (ASHFORD and DIXON, 1935; DICKENS and GREVILLE, 1935; LIPSETT and CRESCITELLI, 1950). However, in the sodium-free medium the usual effect of potassium ions is not observed (CANZANELLI, ROGERS and RAPPORT, 1942; TSUKADA and TAKAGAKI, 1955). These findings were confirmed and details of their relation are presented in Table 1. When glucose was used as a substrate, the glucose utilization, oxygen uptake and lactic acid formation were increased very significantly on the addition of 100 mM-KCl. With pyruvic acid or lactic acid as substrate, the increase of metabolism was also similarly observed. On omission of sodium ions from the medium, however, the addition of potassium ions did not induce any significant changes in the metabolic characteristics, but was somewhat inhibitory.

The effects of adding various amounts of KCl in the medium so as to raise the final concentrations of potassium by 10–100 mM are shown in Figs. 1 and 2. As shown in Fig. 1, the potassium concentration required for the maximal effect was 40 mM with glucose as substrate. Oxygen uptake was already increased significantly on the addition of 20 mM-KCl. In the presence of pyruvic acid as substrate, both oxygen uptake and substrate utilization were significantly increased with a concentration of potassium as low as 10 mM. On further increase of KCl oxygen uptake was increased more, while pyruvic acid utilization was not. This fact shows that the enhancement of pyruvic acid metabolism by adding KCl may not be of a simple nature.

During the above experiments, the medium was made hypertonic by the addition of KCl. However, in the medium which was kept at the same tonicity by omitting the requisite quantity of NaCl when KCl was added, the metabolic responses of cerebral cortex slices to high potassium ion concentrations were quite different. Fig. 3 shows the relation between potassium and sodium ion concentrations and its

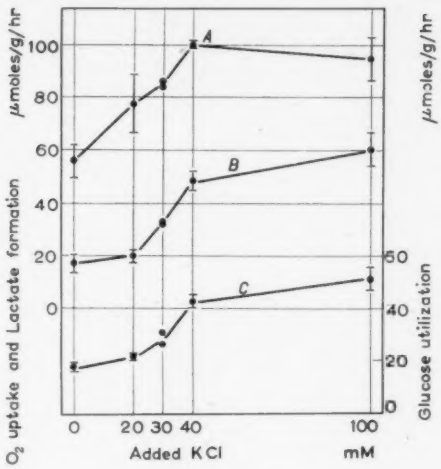

FIG. 1.—Effects of varying concentrations of KCl on glucose metabolism in guinea-pig cerebral cortex slices.

Guinea pig cerebral cortex slices incubated under oxygen; substrate, glucose, 3·3 mM. A, oxygen uptake; B, lactate formation; C, glucose utilization. The mean of five experiments and their standard deviation are shown by point (●) and short bars. The points without short bars show single estimations.

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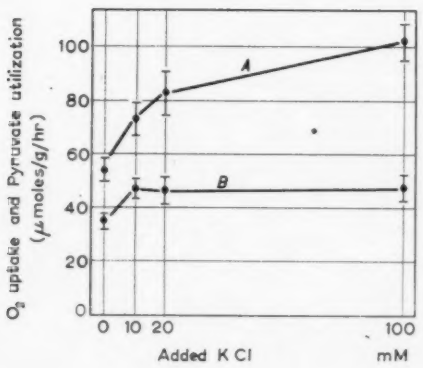

FIG. 2.—Effects of varying concentrations of KCl on pyruvate metabolism in guinea pig cerebral cortex slices.

Guinea pig cerebral cortex slices incubated under pure oxygen; substrate, sodium pyruvate, 6·0 mM. A, oxygen uptake; B, pyruvate utilization. Explanation of data as in Fig. 1.

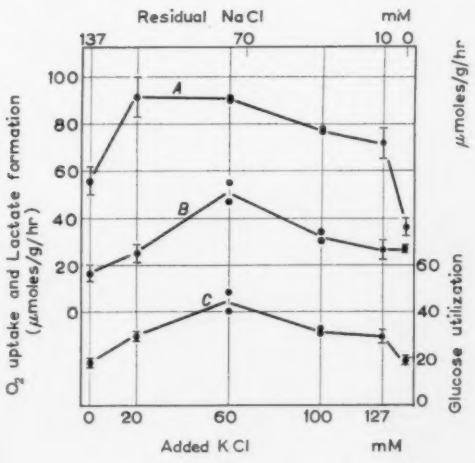

FIG. 3.—Effect of K^+ ions in relation to Na^+ ions.

When varying amounts of KCl were added, the media were kept at the same tonicity by omitting the requisite quantities of NaCl. See text for experimental conditions. A, oxygen uptake; B, lactate formation; C, glucose utilization. Explanation of data as in Fig. 1.

TABLE 1.—THE EFFECTS OF SODIUM-FREE MEDIUM AND K^+ IONS ON THE METABOLISM OF GUINEA PIG BRAIN SLICES

A. Glucose as substrate (3·3 mM)

	Oxygen uptake			Lactate formation			Glucose utilization		
	Control (1)	KCl added* (2)	Change ratio (3)	Control (4)	KCl added (5)	Change ratio (6)	Control (7)	KCl added (8)	Change ratio (9)
Normal control medium	μmoles/g/hr 55·9 ± 6·0† (10)	μmoles/g/hr 94·4 ± 8·1 (4)	(2)/(1) **1·69**‡	μmoles/g/hr 16·6 ± 3·2 (10)	μmoles/g/hr 59·4 ± 5·9 (4)	(5)/(4) **3·58**	μmoles/g/hr 17·7 ± 1·4 (10)	μmoles/g/hr 51·0 ± 4·2 (4)	(8)/(7) **2·88**
Sodium-free medium	58·9 ± 11·7 (6)	45·4 ± 3·3 (7)	0·77	48·8 ± 10·4 (6)	55·3 ± 9·2 (7)	1·13	36·7 ± 5·4 (6)	34·4 ± 2·8 (7)	0·94

B. Pyruvate as substrate (6·0 mM)

	Oxygen uptake			Pyruvate utilization		
	Control (1)	KCl added (2)	Change ratio (3)	Control (4)	KCl added (5)	Change ratio (6)
Normal control medium	μmoles/g/hr 54·9 ± 4·2 (8)	μmoles/g/hr 102·6 ± 6·7 (6)	(2)/(1) **1·87**	μmoles/g/hr 35·3 ± 2·4 (5)	μmoles/g/hr 48·3 ± 4·5 (3)	(5)/(4) **1·37**
Sodium-free medium	93·3 ± 5·2 (5)	77·6 ± 4·4 (6)	0·83	51·9 ± 3·1 (4)	44·7 ± 1·6 (6)	0·86

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C. Lactate as substrate (4·5 mM)

	Oxygen uptake			Lactate utilization		
	Control (1)	KCl added (2)	Change ratio (3)	Control (4)	KCl added (5)	Change ratio (6)
	μmoles/g/hr	μmoles/g/hr	(2)/(1)	μmoles/g/hr	μmoles/g/hr	(5)/(4)
Normal control medium	59·9 ± 3·8 (5)	100·0 ± 3·5 (5)	**1·67**	17·7 ± 2·5 (5)	24·9 ± 2·4 (5)	**1·40**
Sodium-free medium	28·6 ± 4·1 (5)	25·5 ± 3·3 (5)	0·89	—§	—§	

* The concentration of added KCl was 100 mM.
† Mean ± standard deviation. Figures in parentheses give number of experiments.
‡ Figures in bold face type indicate a statistically significant change ($P < 0·01$).
§ No measurable utilization of lactic acid was found.

TABLE 2.—SODIUM-FREE MEDIUM AND THE METABOLISM OF TISSUE SLICES

	Glucose as substrate (3·3 mM)									Pyruvate as substrate (6·0 mM)		
	Oxygen uptake			Lactate formation			Glucose utilization			Oxygen uptake		
	Control (1)	Sodium-free (2)	Change % (3)	Control (4)	Sodium-free (5)	Change % (6)	Control (7)	Sodium-free (8)	Change % (9)	Control (10)	Sodium-free (11)	Change % (12)
	μmoles/g/hr	μmoles/g/hr	$\frac{(1)-(2)}{(1)} \times 100$	μmoles/g/hr	μmoles/g/hr	$\frac{(4)-(5)}{(4)} \times 100$	μmoles/g/hr	μmoles/g/hr	$\frac{(7)-(8)}{(7)} \times 100$	μmoles/g/hr	μmoles/g/hr	$\frac{(10)-(11)}{(12)} \times 100$
Guinea pig brain cortex	55·9 ± 6·0* (10)	58·9 ± 11·7 (6)	+5·4	16·6 ± 3·2 (10)	48·8 ± 10·4 (6)	+194·0	17·7 ± 1·4 (10)	36·7 ± 5·4 (6)	+107·3	54·9 ± 4·2 (8)	93·3 ± 5·2 (5)	+70·0
Guinea pig liver	34·0 ± 3·4 (3)	32·5 (2)	−4·4	<2·0†	<2·0†		—§	—§		46·1 ± 1·7 (3)	40·0 (2)	−13·2
Rat diaphragm	54·8 ± 7·8 (4)	28·7 ± 4·3 (4)	−47·8	9·5 ± 0·9 (4)	8·3 ± 1·2 (4)	−12·5	5·9 ± 1·4 (4)	3·7 ± 2·3 (4)	−37·2	63·9 ± 11·4 (3)	35·8 ± 7·2 (4)	−44·0
Rabbit kidney cortex	80·0 (2)	50·3 ± 1·5 (3)	−37·1	15·8 (2)	8·2 ± 0·1 (3)	−48·1	11·7 (2)	6·4 ± 0·3 (3)	−45·3	94·9 (2)	68·6 ± 2·9 (3)	−27·8

* Mean ± standard deviation. Figures in parentheses give number of experiments.
† The precise value could not be determined by the present method.
§ Glucose content in the medium was slightly increased after the incubation.

effect on the metabolism of cerebral cortex slices metabolizing glucose in the medium maintained at constant tonicity. On increase of potassium ion concentration by 60 mM the metabolic activity of cerebral cortex slices was highest. Above this concentration of potassium ions the metabolism was clearly decreased and on complete omission of sodium ions and replacement with potassium salts, it was lower than in the normal control medium.

As seen in Table 2, in the sodium-free medium the aerobic glycolysis of cerebral cortex slices metabolizing glucose as a substrate was about three times as high as that in the normal control medium and the glucose utilization was twice as high, although the oxygen uptake was not changed at all. When pyruvic acid was used as a substrate, the respiration in the sodium-free medium was almost twice as high as the control. These effects of sodium-free medium on the metabolism of slices metabolizing glucose or pyruvic acid were quite peculiar to brain tissue, and never observed in the slices of liver, diaphragm or kidney. The metabolic activities of slice preparations of the latter tissues were inhibited on omission of sodium ions from the medium. The oxygen uptake of cerebral cortex slices metabolizing lactic acid, citric acid, glutamic acid, succinic acid or fumaric acid was significantly inhibited on omission of sodium ions from the medium (Table 3). The increase of respiration in the sodium-free medium

TABLE 3.—EFFECT OF Na^+-FREE MEDIUM ON THE OXYGEN UPTAKE OF GUINEA PIG CEREBRAL CORTEX SLICES METABOLIZING VARIOUS SUBSTRATES

Substrate and its concentration	Oxygen uptake		
	Control (1)	Sodium-free (2)	Change % (3)
	μmoles/g/hr	μmoles/g/hr	$\frac{(1)-(2)}{(1)} \times 100$
Fructose, 10 mM	55·8 ± 6·7 (4)*	41·4 ± 3·4 (3)	−25·8
Lactate, 6 mM	59·9 ± 3·8 (5)	28·6 ± 4·1 (5)	−52·3
Citrate, 10 mM	58·2 ± 6·5 (4)	27·0 ± 2·7 (4)	−53·6
Glutamate, 10 mM	69·9 ± 3·5 (3)	34·8 ± 4·2 (4)	−50·3
Succinate, 10 mM	73·8 ± 5·7 (4)	50·5 ± 1·2 (4)	−31·6
Fumarate, 10 mM	46·6 ± 2·9 (4)	25·4 ± 2·8 (5)	−45·5
No addition	41·2 ± 4·6 (15)	20·2 ± 0·9 (3)	−51·0

* Mean ± standard deviation; figures in parentheses give number of experiments.

was observed only when pyruvic acid was used as a substrate. When the cerebral cortex slices metabolized fructose aerobically as a substrate, the lactic acid formation was increased to 34·3 ± 0·5 (3) μmoles/g/hr in the sodium-free medium, the control forming only 6·2 ± 0·4 (3) μmoles/g/hr.

These effects of sodium-free medium on the metabolism of cerebral cortex slices are almost analogous to those of high potassium concentration (LIPSETT and CRESCITELLI, 1950).

The omission of potassium ions from the medium caused increases in the glucose utilization and lactic acid formation, but the oxygen uptake was not changed. Also, the metabolism utilizing pyruvic acid as a substrate was almost the same as that in the control medium.

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High concentration of ammonium ions and calcium-free medium

WEIL-MALHERBE (1938) found that 10 mM-NH_4Cl increased the respiration of cerebral cortex slices incubated in the presence of glucose. This was confirmed by GORE and MCILWAIN (1952), although in their case the increase was not as great. In the present experiments (Table 4) the effect was not found to be very significant statistically.

TABLE 4.—EFFECT OF NH_4Cl, Ca^{++}-FREE AND K^+-FREE MEDIUM

Medium	Substrate	Oxygen uptake	Lactate formation	Substrate utilization
		μmoles/g/hr	μmoles/g/hr	μmoles/g/hr
NH_4Cl, 0·01 M	Glucose, 3·3 mM	63·5 ± 5·5 (7)†	**23·7*** ± 2·6 (7)	**24·3** ± 3·2 (7)
	Pyruvate, 6·0 mM	**62·4** ± 5·1 (8)		38·2 ± 2·4 (6)
Ca^{++}-free	Glucose, 3·3 mM	**100·3** ± 7·5 (4)	**31·8** ± 3·1 (4)	**33·5** ± 2·6 (4)
	Pyruvate, 6·0 mM	**80·5** ± 9·8 (7)		**53·6** ± 4·8 (7)
K^+-free	Glucose, 3·3 mM	60·1 ± 5·2 (7)	**24·3** ± 3·6 (7)	**22·5** ± 1·5 (7)
	Pyruvate, 6·0 mM	58·3 ± 3·1 (7)		38·0 ± 4·4 (7)

* Values in bold face type mean statistically significant increase when compared with the control (Table 1), $P < 0{\cdot}01$.

† Mean ± standard deviation; figures in parentheses give number of experiments.

The effect of absence of calcium on the respiration of cerebral cortex slices was investigated by several authors (see ELLIOTT, 1955). In the present experiments, omitting calcium from the medium increased respiration, lactic acid formation and glucose utilization, and also, oxygen uptake and pyruvate utilization where the keto acid served as substrate. These findings show the similarity of the effect of omitting calcium to that of increasing potassium.

DISCUSSION

The maintenance of brain activity *in vivo* depends on the supplies of both oxygen and glucose, and it is therefore believed that the energy required for the brain activity is supplied by the aerobic metabolism of glucose. This may correspond to the facts that brain slices metabolizing glucose or pyruvic acid aerobically are able to maintain a high concentration of phosphocreatine, and also are able to respond metabolically to electrical stimulation (MCILWAIN, 1951*a*, *b*; KRATZING, 1953). The metabolism of cerebral cortex slices is affected by varying the composition of the incubating medium. Of the physiologically important electrolytes, sodium ions have not previously been investigated in respect of their detailed effect on the aerobic metabolism of glucose or pyruvic acid in nervous tissue.

It was observed in the present experiments that the omission of sodium ions from the medium caused significant changes of the metabolic characteristics of cerebral cortex slices. With pyruvic acid as substrate the changes were analogous to those resulting from the addition of potassium ions. But when glucose was used as substrate the oxygen uptake was not increased on omission of sodium ions in spite of the increase in glucose utilization and aerobic glycolysis. These observations are difficult to understand by themselves on the basis of the known metabolic pathways in cerebral tissue. But they might confirm further our previous suggestion that the glycolytic

system and the oxidative system of brain slices can be influenced independently by the ionic composition of the medium (TSUKADA and TAKAGAKI, 1955). The facts that (*a*) the usual effect of high potassium was not observed in the medium lacking in sodium ions, that (*b*) the ratio of the concentration of potassium to that of sodium had an intimate relation to the metabolic characteristics measured, and that (*c*) on omission of sodium ions great changes of metabolism were observed, may suggest a special significance of sodium ions in brain metabolism. Furthermore, the effect of sodium-free medium was found to be peculiar to brain tissue and to be restricted to glucose or pyruvic acid as substrates. These findings may therefore have some bearing on the functional aspects of nervous activity.

The effect of adding 100 mM-KCl to the medium has frequently been studied. At this very high concentration an enhancement of oxygen uptake, lactic acid formation and glucose utilization is readily observed. But this effect of potassium ions is not restricted only to very high concentrations, but also was observed at a relatively low concentration, and not only with glucose as substrate (CANZANELLI, ROGERS and RAPPORT, 1942), but also with pyruvic acid as substrates. The effect of potassium ions might therefore be considered to have some relation to normal physiological phenomena.

On the other hand, the fact that on completely omitting potassium ions the glucose utilization and aerobic glycolysis were increased was surprising. Since the respiration was not changed, this effect appears to be restricted only to glycolysis.

The effects of ammonium ions and of a calcium-free medium are commonly thought to be analogous to the potassium effect. However, in the present study the action of 10 mM-NH_4Cl was not very pronounced. The reason for this divergence from the results of other authors is at present obscure.

It is generally believed that the oxidative metabolism of cerebral cortex slices is enhanced by potassium ions in the presence of sodium, but GORE and MCILWAIN (1952) found that the phosphocreatine content is very low in the presence of a high potassium concentration. Their finding was confirmed by us (TSUKADA and TAKAGAKI, 1954), and the reason for this apparent contradiction should be considered. The lowered phosphocreatine content may be due to the inhibition of its formation, or due to the fact that the increase in its formation is offset by a still greater increase in its rate of breakdown. FINDLAY, MAGEE and ROSSITER (1954) who attempted to resolve these contradictory data, found that a high potassium concentration inhibits the incorporation of ^{32}P into the various phosphate fractions of brain slices. However, under their experimental conditions the usual enhancement of metabolism would not be observed, since sodium ions were almost absent in the medium. The relation between the incorporation of ^{32}P into the phosphate fractions and the metabolic activity of brain slices in media of various ionic compositions has also been reported (TAKAGAKI, HIRANO, SUGIMOTO and TSUKADA, 1956).

SUMMARY

(1) The effect of some inorganic ions on the oxygen uptake, aerobic lactic acid formation and glucose utilization by brain slices was studied. Their effect on the pyruvic acid metabolism was also examined.

(2) The addition of 100 mM-KCl to cerebral cortex slices metabolizing in a sodium-free medium did not cause any increase in the oxygen uptake, lactic acid formation

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or glucose utilization, although in the usual medium it increased the metabolism considerably.

(3) When sodium was omitted from the medium, the oxygen uptake was not changed at all, but the aerobic lactic acid formation and glucose utilization were increased very significantly. The oxygen uptake of cerebral cortex slices was increased on omission of sodium ions only when pyruvic acid was metabolized, and it was never observed when other substrates were used. These findings were peculiar to brain slices and not found in other tissue slices.

(4) The omission of potassium ions caused an increase in glucose utilization and lactic acid formation, while the respiration was not changed.

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METABOLIC REACTIVITY OF BRAIN AND LIVER MITOCHONDRIA TOWARDS CHLORPROMAZINE*

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(*Received* 8 *July* 1957)

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WHEREAS it is undoubtedly difficult and unjustified to base an explanation of *in vivo* drug actions solely on effects obtained by the drug on metabolic processes *in vitro*, drugs may be used as tools in the characterization of metabolic properties of cell organelles such as mitochondria. With this point of view in mind we have studied the action of chlorpromazine (10-(3-dimethylamino-propyl)-2-chlorphenothiazine) on oxidative phosphorylation, in order to establish whether the drug is an uncoupling agent such as 2:4-dinitrophenol, thyroxin and a number of other compounds.

In this paper are summarized the results of a comparative study of the action of chlorpromazine on oxidative phosphorylation in brain and liver mitochondria. It was found that chlorpromazine affected oxidative phosphorylation in liver but not in brain mitochondria when the same experimental conditions were employed. An attempt was made to localize the site of uncoupling along the electron transfer chain in liver mitochondria. In order to explore the reasons for the difference in reactivity of liver and brain mitochondria towards the drug, studies were conducted on the adsorption of chlorpromazine by the cell organelles.

EXPERIMENTAL

Rat liver mitochondria were prepared according to the method of SCHNEIDER (1948) and rat brain cortex mitochondria by a slight modification of this procedure. The cortex of rat brain was homogenized in 0·25 M-sucrose solution and nuclei and cell debris were removed at 1400 G (10 min) in the high speed attachment of the International Centrifuge at 0°. The mitochondrial fraction was obtained by centrifuging the supernatant fluid at 12,000 G for 15 min. The sediment was washed twice by suspension in 0·25 M-sucrose solution. The mitochondrial suspension was made up with 0·25 M-sucrose solution to the original volume of the tissue.

The oxidation was measured by the conventional Warburg technique. Phosphate was determined according to LOWRY-LOPEZ (1946) except in the experiments with cytochrome *c* in which the GOMORI (1941–42) method was used. With ferricyanide as an electron acceptor, the phosphate determination was carried out according to COPENHAVER and LARDY's (1952) modification of KING's method. Ferricyanide

* A preliminary report of this work was published (BERGER *et al.*, 1956). For a review of the biochemical effects of chlorpromazine see BERGER *et al.* (1957) and STRECKER (1956).

Supported in part by grants from the National Institute of Neurological Diseases and Blindness (Grants B226-C and B-557), National Institutes of Health, United States Public Health Service, and the Supreme Council 33° Scottish Rite Masons of the Northern Jurisdiction, United States of America.

disappearance was determined by direct colorimetry at 420 mμ. The procedure of MALEY and LARDY (1955) was employed in experiments in which the phosphorylation coupled to the oxidation of cytochrome *c* was measured. Nitrogen was determined by the Kjeldahl method.

Chlorpromazine was estimated by its absorption in the u.v. at 305 mμ (Beckman u.v. spectrophotometer). In experiments in which the adsorption of chlorpromazine on tissue elements was measured, the drug ($2{\cdot}5 \times 10^{-4}$ M) was incubated at room

TABLE 1.—EFFECT OF CHLORPROMAZINE ON OXIDATIVE PHOSPHORYLATION OF MITOCHONDRIA FROM LIVER AND BRAIN CORTEX (RAT)

	Liver			Brain		
	P (μmoles)	O (μatoms)	P : O	P (μmoles)	O (μatoms)	P : O
α-Ketoglutarate	29·3	10·4	2·8	7·9	2·6	3·0
α-Ketoglutarate + chlorpromazine	21·9	11·6	1·9	6·6	2·2	3·0
Succinate	24·9	11·3	2·2	8·2	5·0	1·6
Succinate + chlorpromazine	12·1	10·5	1·1	7·1	4·6	1·5
Pyruvate + malate	20·7	8·2	2·5	14·7	5·5	2·7
Pyruvate + malate + chlorpromazine	13·2	7·5	1·7	15·1	5·5	2·8
Glutamate	31·3	13·4	2·3			
Glutamate + chlorpromazine	19·8	11·6	1·7			

The reaction vessels contained in μmoles: K phosphate buffer, pH 7·4, 60; $MgSO_4$ 20; ATP 6·5; cytochrome *c* 0·02; sodium fluoride 10–15; substrate; mitochondrial suspension 0·5 ml (average nitrogen, 1·3 mg); the side arm contained glucose 50; and hexokinase (Pabst) 0·4 mg. The flask volume was made up to 3 ml with 0·15 M-KCl or 0·25 M-sucrose. When brain mitochondria were used, DPN at 0·2 mg per flask was added. The following substrate concentrations were used: α-ketoglutarate 30; succinate 30; glutamate 60; pyruvate 60 + malate 3.

Temperature 37° for liver, 30° for brain; incubation 20–30 min in air.

temperature with liver or brain mitochondria and phosphate buffer. The tissue was removed by centrifugation and the amount of chlorpromazine left in the supernatant determined.

RESULTS

Chlorpromazine at a concentration of 2×10^{-4} M partially inhibited the phosphorylation accompanying the oxidation of rat liver mitochondria with α-ketoglutarate, succinate, glutamate and pyruvate + malate as substrates, whereas it did not depress the phosphorylation when mitochondria from rat brain cortex were used and the same experimental conditions were maintained (Table 1). Increasing the concentration of chlorpromazine resulted in the formation of a precipitate which made it impossible to study the oxidative phosphorylation at higher concentrations of the drug. When the brain cortex mitochondrial suspension was diluted and the chlorpromazine concentration maintained, very low oxygen uptake and phosphorylation was obtained and the P/O ratios became meaningless.

Guinea pig and monkey brain cortex mitochondria gave results similar to those obtained with rat brain cortex; the P/O ratios obtained with guinea pig brain

mitochondria were somewhat lower, probably because of a larger amount of ATP-ase in guinea pig brain.

In order to ascertain that the phosphorylation mechanism was not basically different in brain from that in liver the effect on brain mitochondria of 2:4-dinitrophenol at a level of 10^{-5} M was studied with α-ketoglutarate as the substrate. Phosphorylation was inhibited to the same extent as with liver mitochondria.

TABLE 2.—PHOSPHORYLATION COUPLED TO THE OXIDATION OF CYTOCHROME *c* RAT LIVER MITOCHONDRIA

Ascorbic acid (30 μmoles)					
2×10^{-4} M-chlorpromazine					
P (μmoles)	O (μatoms)	P : O	P (μmoles)	O (μatoms)	P : O
5·3	6·5	0·81	3·1	6·9	0·45
3·4	6·8	0·50	0	7·6	0
4·9	4·9	1·0	1·1	5·1	0·22
Adrenaline (10 μmoles)					
5·8	5·8	1·00	0	4·9	0
4·6	5·6	0·82	0	7·4	0

The reaction vessels contained in addition to ascorbic acid or adrenaline: (in μmoles) K phosphate buffer, pH 7·4, 30; ATP 6; $MgSO_4$ 30; NaF 40; cytochrome *c* (Sigma) 0·3; glucose 40; hexokinase 0·4 mg; liver mitochondrial suspension 0·5 ml (average nitrogen 1·7 mg) and sucrose solution (0·25 M) to a final volume of 3·0 ml, gas-phase air, incubation at 30°, with adrenaline for 10 min, with ascorbic acid 20 min.

When cytochrome *c* is oxidized by liver mitochondria in the presence of ascorbic acid or adrenaline, P/O values approaching 1 are obtained (MALEY and LARDY, 1955). In the presence of chlorpromazine the P/O ratios were greatly reduced especially when adrenaline substituted for ascorbic acid with adrenaline as electron donor, no phosphorylation occurred at all in the presence of chlorpromazine (Table 2). Similar results were obtained with low levels of cytochrome *c* with mitochondria previously exposed to hypertonic sucrose (LEHNINGER, 1955). The P/O values in the absence of chlorpromazine varied between 0·5–0·8 and were somewhat lower than the values obtained with the MALEY–LARDY procedure.

When phosphorylation on the cytochrome *c* level was studied with brainm itochondria, little if any phosphorylation was obtained with cytochrome *c*, ascorbate or adrenaline, but oxidation was unimpaired. Because of the possibility that the inability of brain mitochondria to utilize cytochrome *c* for phosphorylation was due to the fact that the commercial cytochrome *c* was derived from tissue other than brain (horse heart), we prepared cytochrome *c* from beef brain by a modification of the method of LOFTFIELD (1955) and tested its utilization by rat brain mitochondria. This cytochrome *c*, as well as commercial horse heart cytochrome *c* (Sigma) subjected to column purification according to the procedure of MARGOLIASH (1954), gave slightly

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higher phosphorylation values than the unpurified commercial cytochrome *c*, but the P/O ratios averaging 0·25 were far below those obtained with liver mitochondria.

It was desirable to find out whether chlorpromazine acts solely on the cytochrome *c* phosphorylation step or also on other parts of the electron transport chain. There is evidence that 2,4 dinitrophenol does not completely depress phosphorylation when α-ketoglutarate is oxidized, approximately 25 per cent of the total phosphorylation

TABLE 3.—EFFECT OF CHLORPROMAZINE ON OXIDATIVE PHOSPHORYLATION RAT LIVER MITOCHONDRIA

Substrate α-ketoglutarate (30 μM); malonate 30 μM
2:4-Dinitrophenol $1·7 \times 10^{-5}$ M*

No. of exp.	O μatoms	P μmoles	P : O	+ 2×10^{-4} M-Chlorpromazine		
				O μatoms	P μmoles	P : O
5	8·1	8·1	1·0	6·7	1·3	0·2

Substrate α-ketoglutarate (30 μM); ferricyanide electron acceptor†

No. of exp.	$\Delta Fe(CN)_6^{\equiv}$ μatoms	ΔP μmoles	P : $2Fe(CN)_6^{\equiv}$	+ 2×10^{-4} M-Chlorpromazine		
				$\Delta Fe(CN)_6^{\equiv}$ μatoms	P μmoles	P : $2Fe(CN)_6^{\equiv}$
9	13·7	21·6	1·3	13·0	19·8	1·3

* Basic flask components as in Table 1.
† The flask contained in addition to α-ketoglutarate: 0·017 M-$NaHCO_3$, 50 μM-K_3FeCN_6, 0·1 γ/flask antimycin A, gas phase 95% N_2—5% CO_2. Incubation at 30° for 20 min.

being retained in a so-called substrate phosphorylation, with a P/O ratio approaching 1 (HUNTER, 1951). The slight residual phosphorylation which remained in the presence of 2:4-dinitrophenol was further suppressed when 2×10^{-4} M-chlorpromazine was added (Table 3).

In recent years specific parts of the electron transfer chain have been studied with the use of artificial electron acceptors. Methylene blue was found to be unsuitable because it depressed phosphorylation and also inhibited oxygen uptake. On the other hand, potassium ferricyanide could be used satisfactorily as an electron acceptor. It has not been established exactly where along the electron chain ferricyanide accepts electrons but there is evidence that this occurs prior to the cytochrome *c* step (COPENHAVER and LARDY, 1952).

With ferricyanide as the electron acceptor and α-ketoglutarate as the substrate, the P/O ratios should approach 2, and in most of our experiments, values between 1 and 2 were found (Table 3). Chlorpromazine at a concentration of 2×10^{-4} M did not affect the P/O values significantly.

Our earlier experiments and also findings by STRECKER (1956) and LINDEN *et al*, (1956) suggested that chlorpromazine is strongly adsorbed* by tissue. It was, therefore, of interest to ascertain whether liver and brain mitochondria differ in the ability to adsorb chlorpromazine, a fact which may account for their different sensitivity toward the drug.

In experiments in which the disappearance of chlorpromazine incubated with

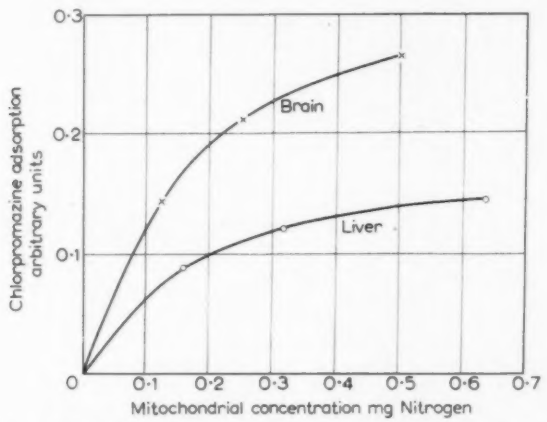

FIG. 1.—*Adsorption of chlorpromazine by mitochondria.* Chlorpromazine ($2{\cdot}5 \times 10^{-4}$ M) was incubated with increasing amounts of liver and brain mitochondria respectively and 40 μM potassium phosphate buffer (pH 7·2) at room temperature for 10 min. The tissue was removed by centrifugation and the chlorpromazine in the supernatant determined by measuring the u.v. absorption at 305 mμ. Corrections were made for the amount of absorption due to tissue alone and the corrected values were subtracted from the optical densities obtained by chlorpromazine controls.

Arbitrary units $= O\,.\,D\,.\,CPZ - [(O\,.\,D\,.\,CPZ + T) - O\,.\,D\,.\,T.]$
where $O\,.\,D\,.\,CPZ$ = optical density of chlorpromazine
T = tissue.

liver or brain mitochondria was determined, the decrease of chlorpromazine in the supernatant fluid after removal of the tissue by centrifugation was much greater with brain mitochondria than with liver particles (Fig. 1).

DISCUSSION

As reported earlier, under our experimental conditions, chlorpromazine uncoupled the phosphorylation accompanying the oxidation of different tricarboxylic acid cycle substrates with liver mitochondria but not when brain cortex mitochondria were used (BERGER, 1955, 1956; BERGER *et al.*, 1956, 1957). We did not attempt to explore the conditions under which chlorpromazine would uncouple the oxidative phosphorylation of brain mitochondria. Such conditions probably exist, since ABOOD (1955) found decreased P/O values in the presence of chlorpromazine with brain mitochondria. Similarly, BERNSOHN *et al.* (1956) report an inhibition by chlorpromazine of P/O values of brain mitochondria. The P/O ratios of the controls in the experiments of BERNSOHN *et al.* are extremely low and the lowest chlorpromazine level to give a slight inhibition of P/O values exceeds by far the drug concentration

* There is evidence that the disappearance of chlorpromazine is due to its adsorption on to tissue since the drug could be partially recovered by extraction with organic solvents (STRECKER, 1956).

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in absolute terms and relative to the mitochondrial concentration used in our experiments. We did not want to surpass this concentration of chlorpromazine since we found the formation of a precipitate at higher concentrations. It is of considerable interest to compare the drug action on liver and brain particles under specific conditions in order to get a clue not only to the drug mechanism but also to some basic metabolic differences between the two tissues.

It cannot be assumed at present that the effect of chlorpromazine on depressing P/O values obtained with liver mitochondria reflects the *in vivo* action of the drug. Nevertheless, it was hoped that by studying parts of the electron transport system some evidence may be obtained as to whether the drug may act on a single phosphorylation step. Even though no significant inhibition of oxidative phosphorylation could be observed when ferricyanide was the electron acceptor, the fact that both the substrate phosphorylation of α-ketoglutarate in the presence of 2:4-DNP and the phosphorylation associated with the oxidation of cytochrome *c* was inhibited by chlorpromazine strongly suggests that the drug does not act on one single phosphorylation step, but probably non-specifically on different enzymes or enzyme systems to various degrees (cf. STRECKER, 1956; BERGER *et al.*, 1957).

Although the greater adsorption of chlorpromazine by brain than by liver mitochondria would make one expect that the drug may also inhibit metabolic processes to a greater extent in the former, one may speculate that this greater adsorption capacity protects the essential structure of brain mitochondria responsible for oxidative phosphorylation, while in the liver particles it is exposed to the action of the drug.

It appears that the different sensitivity of the mitochondria of the two tissues is determined rather by the structural differences than by some intrinsic differences in metabolic pattern, although brain mitochondria are supposed to contain the glycolytic systems in contrast to liver mitochondria (HASSELBACH *et al.*, 1953; GALLAGHER *et al.*, 1956). The lack of phosphorylation obtained when exogenous cytochrome *c* is oxidized by brain mitochondria also supports the concept of structural differences between the particles from the two tissues.

Evidence for the importance of the structure of mitochondria and the organization of enzyme systems responsible for oxidative phosphorylation may be seen in the observation that thyroxin, although uncoupling oxidative phosphorylation in intact liver mitochondria does not do so with digitonin treated mitochondria which still carry out oxidative phosphorylation (COOPER and LEHNINGER, 1956). This is further supported by findings that the hormone does not uncouple oxidative phosphorylation when brain mitochondria are used (LARDY, 1956).

SUMMARY

(1) Chlorpromazine at a concentration of 2×10^{-4} M partially uncoupled the phosphorylation accompanying the oxidation of several tricarboxylic acid substrates with rat liver mitochondria whereas it failed to do so under the same conditions with rat brain cortex mitochondria.

(2) An attempt was made to localize the site of action of the drug along the electron transport chain, but it appears that chlorpromazine does not act specifically on any one site along the chain.

(3) Brain mitochondria have a greater ability to adsorb chlorpromazine than liver mitochondria.

Acknowledgements—I wish to thank Prof. HEINRICH WAELSCH for his encouragement and advice. I also wish to thank Dr. HAROLD STRECKER for his interest during the course of this work.

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Journal of Neurochemistry, 1957, Vol. 2, pp. 37-44, Pergamon Press Ltd., London

CYTOCHROME OXIDASE IN HUMAN BRAIN TUMOURS*

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(*Received* 25 *May* 1957)

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THE investigation reported in this communication has been undertaken for two principal reasons. The first aim has been to estimate the activity of an enzyme important to biological oxidations in a series of gliomas of varying types and degrees of malignancy, correlating such data where possible with histological characteristics. The second purpose has been to obtain some indirect evidence relative to the oxidative metabolism of normal glia from the study of well differentiated gliomas.

The measurement of enzyme activities in human brain tumours involves some inherent difficulties of considerable magnitude. Care must be exercised to assure the utilization of intact viable neoplastic tissue, free from the frequently accompanying areas of haemorrhage and necrosis. Since the enzymic characteristics of grey matter may differ widely with those of certain tumours, one must restrict one's studies to samples of tumour tissue freed from the contaminating influences of invaded cortex or basal ganglia. Moreover, since it is commonplace to find brain tumours of more than one cell type, with the composition varying literally from one millimetre to the next, it would be most desirable to have histological controls closely approximated to the sample upon which the enzyme assay is being carried out. For these reasons the Linderstrøm-Lang technique of quantitative histochemistry was used, with the hope that as true a series of measurements as possible might be obtained.

Cytochrome oxidase was selected for study because this enzyme is reported to be generally influenced by the process of malignant transformation. Further, since the respiration of a number of gliomas has already been reported (VICTOR and WOLF, 1937; HELLER and ELLIOTT, 1955), comparison of these data with levels of cytochrome oxidase would be of interest.

MATERIAL AND METHODS

Samples of intracranial tumours and resected brain tissue were obtained directly at operation, placed immediately in an ice carrier and transported to the laboratory. The specimens were quickly dissected free of haemorrhagic and grossly necrotic areas. Representative blocks were frozen and adjacent blocks were fixed and stained with standard chromatic and metallic stains. Frozen specimens were stored in dry ice until enzyme determinations were performed.

Following study of the histological preparation, blocks of anticipated homogeneity and good viability were selected and prepared for section. The technique of preparation was that of POPE (1952). From a cube of frozen tissue, a cylinder was prepared by means of a tissue punch of 2·5 mm internal diameter. The remaining portion of the cube was thawed, marked on its upper surface with India ink, and fixed in alcohol formalin. Longitudinal sections of this block were used to help in orientation

* This investigation was supported in part by Grant No. 119 from the National Multiple Sclerosis Society.

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and identification of the tissue. The cylindrical block was affixed to a precision rotary microtome in a cold room at $-12°$ and serially sectioned at a thickness of 40 μ. The first section was placed on an albuminized slide to be used as a histological control. Eight sections were placed together in a glass microhomogenizer for the enzyme determination. Four sections taken at intervals along the preceding samples were saved in a desiccator for dry weight determinations. From two to seven series of sections as described were obtained from a single cylindrical block. Blocks were studied from several different portions of each tumour.

The sections retained for histological study were thawed on the albuminized slide, fixed in alcohol formalin and stained with thionin. After histological observations were completed, nuclear counts were performed on each section. This was done by means of an ocular reticule micrometer having an area of 0·0538 mm^2 at 430 magnification. Total nuclei per reticule were counted in nine fields of the sections and results expressed as thousands of nuclei per mm^3 of tissue. Since the ratio of nuclear thickness to thickness of the tissue was small, the correction for transected nuclei discussed by ABERCROMBIE (1946) was not used. A correction for shrinkage after fixation in the horizontal dimensions was of greater magnitude and was employed. To obtain this correction, diameters were directly measured on the frozen cylinder with a vernier caliper and on the stained section with a stage micrometer. Crude counts were multiplied by the factor A/A', were A = area of stained section and A' = area of the unfixed frozen section.

The technique for determination of cytochrome activity was that of HESS and POPE (1953). All operations were carried out in a cold room at 4°. Eight microtome sections were used per determination because of the generally low activities encountered in most of these tumours. The sections were suspended in 30 μl of 0·75 per cent sodium desoxycholate in an iced microhomogenizer and homogenized for 1 min. Speed of homogenization was a critical factor. The optimal speed for the tissues used under these conditions was 1200 rev/min and this speed was used throughout. The homogenate was centrifuged at 2500 rev/min for 10 min. Five μl of the supernatant were added to 65 μl of a $2{\cdot}5 \times 10^{-5}$ M solution of reduced cytochrome c in 0·05 M disodium phosphate buffer, pH 7·1, in a quartz microcuvette. The rate of decrease in optical density at a wave length of 550 μ was measured in a Beckman DU spectrophotometer. Duplicate determinations were done on all samples. The rate of oxidation of reduced cytochrome c was derived from the zero order portion of the curve and results were expressed as decrease in optical density/μg fat-free dry weight $\times 10^3$.*

Sections reserved for weighing were dried in a desiccator and weighed on an Ainsworth microbalance. Total lipid extraction was performed by immersing each section in a 2:1 chloroform-methanol mixture for 10 min followed by another 10 min immersion in toluene (POPE, 1954).

RESULTS

The unit of reference chosen for expression of results has been the fat-free dry weight. The total dry weight has not been used because of the occasional presence in some gliomas of free lipid secondary to myelin destruction. Cell counts have been done to indicate cell densities, however, the extreme variation of cell volume in different tumours renders comparisons of enzyme activity on a per cell basis of little value. The ideal standard of reference would be total cytoplasmic volume of tumour cells. Of the three units employed here, the fat-free dry weight should provide the closest approximation to that optimal unit of reference.

Early in the course of this study, the elimination of portions of grey matter was found to be of critical importance. Despite efforts to select desirable blocks of tissue for analysis, it was occasionally found upon examining the histological controls that unsuspected areas of infiltrated cortex were present. An example of this in an invasive protoplasmic astrocytoma is shown in Table 1. In series one, a large number of tumour cells were present and cytochrome oxidase activity was approximately a quarter that of intact cortex. In the succeeding series the proportion of nerve cells steadily diminished and the number of neoplastic astrocytes was considerably

* This expression may be directly converted to μmoles cytochrome c oxidized per mg dry wt. per hr at 22° by directly multipliyng the above expression by the factor 214 (HESS and POPE, 1953).

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TABLE 1.—ASTROCYTOMA. RELATION OF CYTOCHROME OXIDASE TO NERVE CELL CONTENT

Series	Depth in cylinder (μ)	Tumour nuclei per mm^3 $\times$ 10^{-3}	Neuronal nuclei per mm^3 $\times$ 10^{-3}	Cytochrome oxidase ΔOD*/min/μg fat-free dry wt. $\times$ 10^3
1	520	27·5	5·6	1·34
2	1040	21·3	5·2	1·23
3	1560	34·2	3·3	1·10
4	2080	48·5	3·1	0·72
5	2600	43·1	1·7	0·60
6	3120	57·3	1·5	0·47
7	3640	75·0	0·43	0·42

*OD = optical density

TABLE 2.—TOTAL SOLIDS, LIPIDS, CELL DENSITIES, AND CYTOCHROME OXIDASE ACTIVITIES IN HUMAN BRAIN TUMOURS

Specimen		Total solids (mg per 100μl)	Extractible lipid (mg per 100 μl)	Nuclei per mm^3 $\times$ 10^{-3}	Cytochrome oxidase ΔOD/min/μg fat-free dry wt. $\times$ 10^3
Cerebral cortex	BT–8	16·2	5·2		4·50
Cerebellar cortex	BT–17	18·8	7·7		1·95
White matter	BT–8	24·9	18·0		0·64
Astrocytoma, protoplasmic	BT–1	12·9	6·6	53	0·68
Astrocytoma, protoplasmic	BT–16	15·0	5·7	75	0·42
Astroyctoma, protoplasmic	BT–18	25·3	15·2	100	0·27
Astrocytoma, fibrillary	BT–9	15·3	2·8	367	0·39
Oligodendroglioma	T–11	10·6	3·8	167	3·09
Glioblastoma	BT–2	13·3	3·9	27	1·06
Glioblastoma	BT–12	16·0	3·8	342	1·12
Glioblastoma	BT–13	13·3	4·7	138	0·97
Glioblastoma	T–10	12·7	3·2	50	1·36
Astrocytoma-oligodendroglioma (mixed)	BT–14	15·6	4·5	114	0·81
Glioblastoma-oligodendroglioma (mixed)	BT–10	14·6	3·7	199	1·06
Meningioma	BT–6	14·8	4·3	348	0·30
Meningioma	BT–7	14·2	3·3	289	1·10
Meningioma	BT–15	15·5	4·1	218	0·50
Meningioma	T–2	16·0	2·7		1·06
Acoustic neurinoma	BT–17	13·0	1·9	131	0·22

increased. Cytochrome oxidase varied approximately in proportion to the content of neurons, reaching a very low level in the final series composed predominantly of astrocytoma. Such experiments are of some interest in noting qualitative differences between some of the gliomas and cortical tissue, but are of little value in quantitation. All determinations in which the histological control revealed the presence of invaded grey matter or necrosis have, therefore, been excluded from this report, leaving a total of sixteen tumours with eighty-two determinations.

Table 2 includes the mean values of data obtained on these specimens. The normal controls were limited to resected portions of brain found to be free from damage due to oedema, traumatization, or compression. The sample of cerebellum consisted of a block of normal cerebellum containing molecular and granular layers and some adjoining white matter. Samples of cortex and white matter were histologically normal blocks from the left middle frontal gyrus and underlying white matter. Results on these latter two samples compare favourably with the mean figures derived from a large series of determinations on frontal lobe biopsies by POPE *et al.* (1957). When calculated on a fat-free reference, POPE's mean values were for cortex 5·23 as compared with my own figure of 4·50, and for white matter 0·65 compared with my figure of 0·64.

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The total solid and extractible lipid contents of most of these tumours tends to be lower than the respective content of normal brain. The lowest values for lipid were found in the fibrillary astrocytoma and the acoustic neurinoma, doubtless related to the dense glia fibrils and collagen fibres found in these two tumours respectively. The protoplasmic astrocytoma BT-18 contained a remarkably large quantity of lipid. This tumour, however, had invaded white matter leaving intermingled among the tumour cells a rather large quantity of free fat demonstrable on fat stain. The oligodendroglioma showed a rather large proportionate content of lipid, the total extractible lipid averaging 36 per cent of total solids.

The cytochrome oxidase determinations on the gliomas are shown in greater detail in Fig. 1, where individual determinations are plotted and the degree of scatter shown. Values for all the well differentiated astrocytomas have been very low, approximating that of white matter and averaging less than 10 per cent of the activity found in cortex. Of particular interest have been the results found in a single, typical, well differentiated oligodendroglioma. These values have been much higher than those found in any other tumour studied, with a sevenfold increase over the astrocytomas, and about 70 per cent of the activity of normal cortex. In four glioblastomas activities ranged from 0·80 to 1·88, most of the points being well above those of astrocytoma but much lower than the range for oligodendroglioma. Two mixed gliomas are also included in Fig. 1. The first was a well differentiated protoplasmic astrocytoma containing a small component of neoplastic oligodendroglia. Values for this specimen tended to be higher than those of the astrocytoma, the mean figure being twice as great. Another tumour was composed of glioblastoma containing small clusters of neoplastic oligodendroglioma in varying proportions. Cytochrome oxidase activity on this specimen fell within the range of the other glioblastomas, although it was of interest to note after examination of histological controls that samples containing larger numbers of oligodendroglia conformed to the four highest points.

Five benign intracranial tumours have been included in this series (Table 2). Four meningiomas, all of similar appearance and histologically benign, were found to

have moderately low activities. The variations noted within this group could not be correlated with histological characteristics. A single acoustic neurinoma had the lowest activity of all the tumours studied.

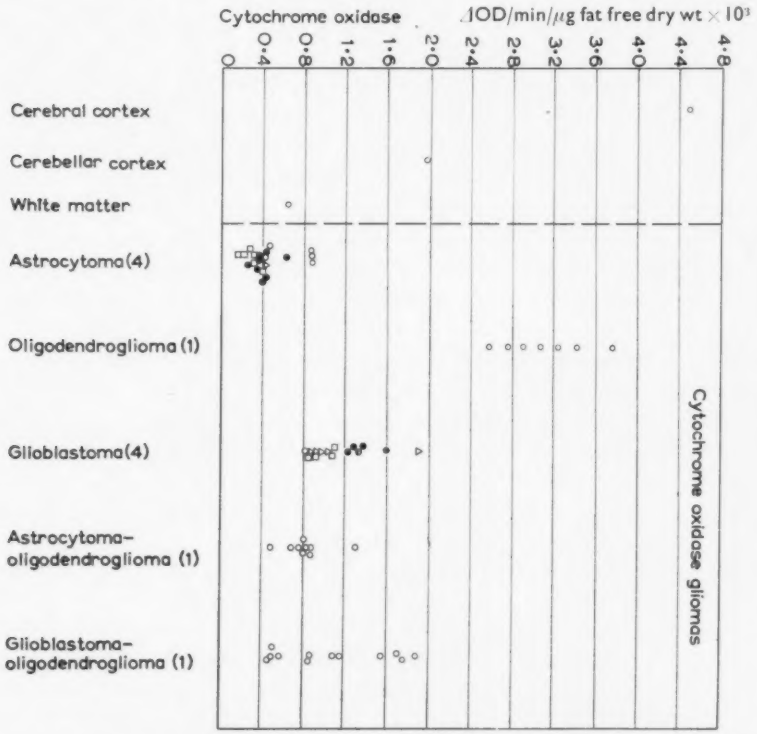

FIG. 1.—Individual determinations of cytochrome oxidase activity are plotted for various gliomas. Mean values for normal brain are included for comparative purposes. Numerals in parentheses refer to the number of separate specimens of the particular tumour type. In cases in which more than one example of a tumour type was studied, separate graphic characters are used to designate the individual specimens.

COMMENTS

Cytochrome oxidase activities have been reported in a large number of experimental and human neoplasms (GREENSTEIN *et al.*, 1943; 1944; SCHNEIDER and POTTER, 1943). Early work on metabolism of tumours had suggested that oxygen consumption and respiratory pigments were low in malignant neoplasms (WARBURG, 1956). Recent studies of CHANCE *et al.* (1952; 1956) leave little doubt but that enzymes concerned with electron transport may be present in malignant tissue in sufficient quantity as not to constitute a limiting factor to cellular respiration. A comparison of cytochrome oxidase activity in tumours against the range of activities in a variety of normal tissues, does, nevertheless, reveal an interesting trend. In general, it has been found (GREENSTEIN *et el.*, 1944; SCHNEIDER and POTTER, 1943) that the values for malignant tumours are much lower than those for normal tissues having high oxidative metabolism (heart, kidney, brain) but are higher than those for normal tissues with low oxidative metabolism (pancreas, intestinal mucosa and skin). In

respect to cytochrome oxidase, then, all tumours studied may be said to occupy an intermediate position between the extremes of variation of normal tissues. A few studies have been done directly comparing tumours of various grades of malignancy with the tissues of derivation. In the case of mouse and rat hepatomas of experimental origin, the primary hepatoma has distinctly lower activity than the normal liver, and the transplanted hepatoma has even lower values (GREENSTEIN *et al.*, 1943; SCHNEIDER and POTTER, 1943). In a few other examples, such as mouse lymph node and mouse lung, the respective tumours show lower values than the tissues of origin (GREENSTEIN, 1954). In the case of tissues normally containing only minimal amounts of cytochrome oxidase, however, there is a departure from this trend. In intestinal carcinomas of mouse and man, cytochrome activity may be higher than in normal intestinal mucosa (GREENSTEIN *et al.*, 1944). In the study of CARRUTHERS and SUNTZEFT (1947) on epidermal carcinogenesis in mice, cytochrome oxidase rose during initial stages of the process, dropped at the stage of carcinoma, but still remained higher than levels in normal skin.

In the present study, cytochrome oxidase in the most malignant tumours (glioblastomas and mixed glioblastoma) have, indeed, been found to be approximately intermediate between a tissue of high activity (cortex) and one of very low activity (white matter). Among the gliomas as a group, however, are to be noted some specific differences which warrant particular attention.

Of especial interest has been the remarkably high values obtained in the well differentiated oligodendroglioma. These figures are of the order of 0·67 that of normal cerebral cortex and are sevenfold higher than the mean values for the well differentiated astrocytomas. These results parallel the observed oxygen consumption of similar human gliomas. VICTOR and WOLF (1937) were the first to study metabolism of these neoplasms. Their series contained two oligodendrogliomas both of whose oxygen uptakes were eleven times greater than the mean value for the astrocytomas. HELLER and ELLIOTT (1955) have more recently reinvestigated this problem, finding the oxygen consumption of a single oligodendroglioma to show a 6·1-fold increase over that of the mean for several astrocytomas. An exception to these findings appears in the recent communication of BRIERLEY and MCILWAIN (1956) who report the oxygen consumption of an oligodendroglioma as being about the same as that of other gliomas. This difference from the previously reported results may conceivably be related to the fact that the neoplasm studied by these authors was not a completely typical oligodendroglioma.

The conclusion seems inescapable, then, that tumours composed of typical neoplastic oligodendroglia have a higher capacity for oxidative metabolism than tumours made up of neoplastic astrocytes. It is unlikely that this characteristic is a manifestation of the process of neoplasia itself. The magnitude of the observed cytochrome oxidase activity is much greater than in any other type of tumour reported in the literature. It would be much more reasonable to consider that the normal oligodendrocyte has a relatively high oxidative metabolism and that the oligodendroglioma shares this property to a comparable degree. Such a conception would not be a new one, since for many years neuropathologists have observed oligodendrocytes to be more sensitive to various anoxic states than are astrocytes.

There is, of course, a discrepancy between the value for oligodendroglioma and that for white matter whose predominant cell is the normal oligodendrocyte. This

discrepancy may be largely an artefact of the unit of reference in comparing these dissimilar tissues. The tumour is composed of fairly uniform cells which are numerous and densely packed. White matter, on the other hand, contains oligodendrocytes whose total cytoplasmic volume must be small in relation to the great mass of myelin and axoplasm. If the oligodendrocyte were the principal repository of cytochrome oxidase in the white matter, then its contribution could be greatly diluted by less active structures. A comparison of tumour and normal white matter on a per cell basis would, therefore, be pertinent. Oligodendrocytes were counted in the sample of white matter, and it was assumed that all the cytochrome oxidase activity of white matter was attributable to these cells. When the specific activities were expressed as decrease in optical density per minute per thousand nuclei $\times 10^3$, the value for neoplastic oligodendrocytes was 0·21 and for normal oligodendrocytes 0·14. Such values are of a similar order of magnitude and indicate that it is at least conceivable that normal and neoplastic oligodendrocytes may contain similar concentrations of cytochrome oxidase.

Cytochrome oxidase activities in the astrocytomas were uniformly low, with no differences found for protoplasmic or fibrillary types. This again correlates well with the observation on oxygen consumption of astrocytomas by VICTOR and WOLF (1937) and HELLER and ELLIOTT (1956). POPE *et al.* (1957) have recently presented evidence indicating that normal astrocytes contribute very little to the cytochrome oxidase of human and rat cortices. The present data on well differentiated astrocytomas would support that conclusion.

The glioblastomas proved to be more active than the astrocytomas, but much less so than the oligodendroglioma. Once again this parallels the observations of VICTOR and WOLF (1937) and HELLER and ELLIOTT (1956), both groups finding the oxygen consumption of the glioblastoma multiforme to be intermediate between astrocytoma and oligodendroglioma. In its metabolic, as well as its histological properties, then, the glioblastoma may be said to occupy a common point between the extremes of the specialized differentiated glial cells.

Although not the primary concern of this report, some note can be taken of the single acoustic neurinoma in this series. This particular specimen appeared typical of the group and was composed of Antoni type *A* cells. The evidence of neuropathology has still failed to prove conclusively whether this tumour originates from the Schwann cell or the perineural fibroblast. Electron microscopy has demonstrated the role of the Schwann cell in formation of myelin in peripheral nerve (GEREN, 1954). Recent work by FOSTER (1956) strongly suggests that the sheath of Schwann in squid nerve has an active oxidative metabolism. It might be expected, therefore, that neoplastic Schwann cells in an acoustic neurinoma would partially reproduce the functions of the normal cell. Observation on the tumour in this series show no such tendency. The cytochrome oxidase proves to be the lowest of the entire group of tumours. In support of this finding, VICTOR and WOLF's series (1937) contained two similar tumours described as perineural fibroblastomas whose oxygen uptakes were of the same order as astrocytoma. In the presently reported tumour, the extractible lipid was also low, only 15 per cent of the total solids. These findings would suggest one of two conclusions: either this tumour is not composed of Schwann cells, or human neoplastic Schwann cells behave differently from anything one might expect from their properties in lower animals.

SUMMARY

Cytochrome oxidase activities have been measured by the method of quantitative histochemistry in sixteen human intracranial tumours and three samples of normal brain. Results have been related to the histological characteristics of the neoplasms. Particular attention has been directed toward restricting the reported samples to typical viable specimens free of necrosis or invaded cortex. The well differentiated astrocytomas have shown very low activities of cytochrome oxidase, even lower than that of white matter. A single, typical oligodendroglioma was found to have very high activity, about two-thirds that of cortex. The glioblastomas occupied an intermediate position. These data closely parallel the reports of others on oxygen consumption of gliomas.

It is suggested that the differences in oxidative metabolism of the oligodendroglioma and astrocytoma reflect differences in the properties of the respective normal cells.

Acknowledgements—The author wishes to thank Dr. ALFRED POPE for his kind interest and advice in this work. He also expresses his gratitude to Drs. BERTRAM SELVERSTONE, WALTER WEGNER, WARREN SISSON, and BARNES WOODHALL for generously providing tissue specimens.

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GLYCOGEN SYNTHESIS IN BRAIN CORTEX SLICES AND SOME FACTORS AFFECTING IT

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(*Received* 29 *April* 1957)

A NUMBER of physiological processes are associated with changes of the glycogen level in the brain. The observation of LEBARON (1955) that brain cortex slices synthesize glycogen *in vitro* made it possible to study this metabolic process more closely, and to examine some factors affecting it.

The effect of increased concentrations of potassium ions on glycogen synthesis was examined, in view of its known action on the Pasteur effect in brain tissue (ASHFORD and DIXON, 1935; DICKENS and GREVILLE, 1935). At the same time changes of water and cation concentrations in the slices, brought about by varying concentrations of potassium ions in the medium, were studied. Since glutamic acid is known to play an important role in brain metabolism and to affect cation transport (KREBS, EGGLESTON and TERNER, 1951) and synthesis of adenosinetriphosphate (ACS, BALAZC and STRAUB, 1953), it was of interest to examine the effect of this amino acid, and of aspartic acid, on glycogen synthesis. Finally, experiments are reported on the effect of some cholinesterase inhibitors on glycogen synthesis and the energy metabolism of brain cortex cells, using selective inhibitors of both true acetylcholinesterase and pseudo-cholinesterase (BAYLISS and TODRICK, 1956). Since it is known that some agents blocking cholinesterases affect electrolyte transport across cellular membranes (USSING, 1954; STRICKLAND and THOMPSON, 1955; STRICKLAND, THOMPSON and WEBSTER, 1956), the mechanism of action of these substances on the transport of cations and water in brain cortex slices was also examined.

METHODS

Guinea pigs of 350 g average weight, were used except in one series of experiments with varied K^+ concentration, where rabbits were used. The brain was removed and prepared as described by MCILWAIN (1951), and slices of the brain cortex were then prepared by the method of DEUTSCH (1936). The first three slices of each hemisphere, weighing between 70 and 100 mg, were used for incubation. Experiments were started not later than 20 min after killing the animal.

Experimental conditions for studying glycogen synthesis

Slices were incubated in a Warburg apparatus for 3 hr at 37° in 25 ml Erlenmeyer flasks, closed with a rubber stopper with two tubes for admitting gas mixtures. Oxygen was used as the gaseous phase. Krebs-Ringer phosphate saline pH 7·4 (2 ml) containing 0·4% glucose was used as the standard medium. Where the effect of K^+ was examined, this medium was varied by replacing the appropriate amount of 0·154 M-sodium chloride with an equivalent amount of isotonic potassium chloride. Glycogen was estimated essentially as described by KERR (1936), but to 1 ml of alkaline hydrolysate, 0·2 ml of a saturated solution of sodium sulphate was added (SJÖGREN, 1938), and cerebrosides were extracted with carbon tetrachloride instead of chloroform. It was found that commercial chloroform often contains impurities reducing the SOMOGYI (1952) reagent, which are difficult to

remove. After hydrolysis, reducing sugar was estimated colorimetrically by the method of SOMOGYI (1952) and NELSON (1944). The S.E. of glycogen estimations in duplicate slices of brain cortex was ±8 per cent. All results presented are expressed as μg glucose/g fresh tissue.

Lactic acid in the medium was estimated by the method of HULLIN and NOBLE (1953). The S.E. of lactic acid estimations on duplicate slices was ±6 per cent. All results are expressed in mg/g fresh tissue. The values of glycogen and lactic acid presented in the tables are the mean of two duplicate estimations.

Manometric experiments

Slices were incubated at 37° for 90 min in conventional Warburg vessels. Where oxygen uptake was measured, 3 ml of phosphate saline pH 7·4, were placed in the main compartment, and 0·2 ml 2N-KOH in the centre well. Where $Q^{O_2}_{CO_2}$ and $Q^{N_2}_{CO_2}$ were measured, 3 ml of bicarbonate saline (KREBS and HENSELEIT, 1932) were used as medium. The gaseous phase for $Q^{O_2}_{CO_2}$ measurement was a mixture of 5% CO_2 and 95% O_2, for the measurement of $Q^{N_2}_{CO_2}$ a mixture of 5% CO_2 and 95% N_2. Anaerobic conditions were secured by placing a stick of yellow phosphorus in the centre well. All media contained 0·4% glucose as substrate. Results are expressed using conventional metabolic quotients.

Transport of cations and water

In experiments in which the permeability of brain cortex cells for the passive transport of cations and water was studied, slices were analysed immediately after preparation (initial values I), or leached for 2 hr at 0° in a mixture of 10 vol of 0·154 M-NaCl and 1 vol of 0·1 M-Na phosphate buffer, pH 7·4, with or without inhibitor (T_0). The tissue was then gently blotted on a sintered glass slide (MCILWAIN, 1951), weighed on a torsion balance and dried overnight at 95°. From the difference of weights of wet and dried tissue the water content (ml H_2O/100 g dry weight) was calculated. The dry tissue was then transferred to test tubes, 1 ml concentrated nitric acid was added, and after 48 hr digestion at room temperature the sodium and potassium contents were estimated in the diluted digest, using a single-ended Zeiss flame photometer with interference filters. All values presented are the mean of four experiments, each performed in duplicate. The mean error of these estimations did not exceed 2·1 per cent.

Where the effect of agents blocking cholinesterases on the transport of cations and water was examined, slices were leached at 0° in 0·154 M-NaCl for 2 hr, and then incubated for 30 min at 30° in 3 ml phosphate saline, containing 0·4% glucose and the inhibitor. Slices incubated in the same medium without inhibitor served as controls. After incubation the contents of water, sodium and potassium were estimated as described above. In these experiments initial values (I), values after leaching (T_0) and after 30 min incubation (T_{30}) were measured.

The initial values of water, sodium and potassium shown in the tables differ slightly from those usually given for brain cortex, since before weighing the slices were rapidly rinsed in 0·154 M-NaCl. The values for sodium and potassium were not corrected as described by PAPPIUS and ELLIOTT (1956*b*), these corrections being rather small.

Materials used. Lysivane (N-[2-(methyl, diethylamino) ethylene]-dibenzothiazine) a selective inhibitor of pseudo-cholinesterase (BAYLISS and TODRICK, 1956) was a gift from May and Baker, Ltd., Dagenham, England. Compound 284 C 51 j, (1:5-bis-(4-allyldimethylammonium phenyl) pentane-3-one-dibromide) was a gift from Dr. F. C. COPP, Burroughs Wellcome Research Laboratories, Langley Court, England. Other substances used were commercial preparations.

RESULTS

(1) *Effect of K^+ ions on glycogen synthesis in brain cortex slices*

As shown in Fig. 1, increased concentrations of K^+ ions brought about an inhibition of glycogen synthesis. An inhibitory effect was found at concentrations of 50 mM-K^+, whereas LEBARON (1955) observed at concentrations of 45 mM a slight stimulation which, however, did not appreciably exceed the experimental error. Within the concentration range of 6·4 mM (i.e. the concentration of K^+ in standard phosphate

saline) to 40 mM-K^+ no differences in the rate of glycogen synthesis were found in our experiments. The decrease of glycogen synthesis was always accompanied by an increase in lactic acid formation. In absence of potassium no glycogen was formed.

The rate of glycogen synthesis observed in our experiments was somewhat lower (244 ± 17 μg glucose/g wet weight/3 hr; 26 observations), than the values reported by LEBARON (about 410 μg glucose/g wet weight/3 hr). No explanation can be offered for this discrepancy; it should be pointed out, however, that considerable differences were observed between the initial values in our experiments (260 ± 9 μg/g;

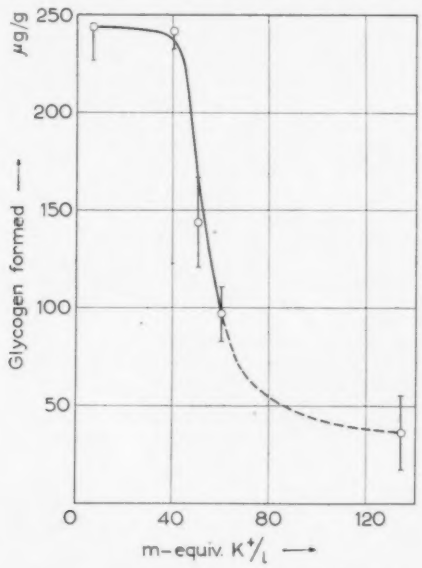

FIG. 1.—The effect of varying concentrations of K^+ on glycogen synthesis in brain cortex slices. Slices incubated 3 hr at 37° in phosphate saline containing 0·4% glucose; O_2 as gaseous phase. Distribution marks are ±S.E., each point having been calculated from at least seven estimations, made in duplicate. Formed glycogen expressed in μg glucose/g wet weight/ 3 hr.

36 observations) and those of LEBARON (72 ± 36 μg/g), while the levels of glycogen attained after 3 hr incubation did not differ appreciably. Possibly the state of nutrition and age of the experimental animals may account for this difference.

It is known that in the presence of glucose cells maintain under aerobic conditions a concentration gradient of K^+ against the medium, and the intracellular concentration of K^+ may reach levels higher than 100 m-equiv./l. tissue fluid (PAPPIUS and ELLIOTT, 1956*b*). Thus an increase of K^+ concentration in the medium has little influence on its intracellular concentration. The effect of varying concentrations of K^+ in the medium on the contents of water and cations in brain cortex slices was therefore examined, both during leaching at 0° in 0·154 M-NaCl, and during 30 min incubation in phosphate saline at 30°. Work on some aspects of this question was recently published elsewhere (PAPPIUS and ELLIOTT, 1956*a*, *b*).

As shown in Figs. 2 and 3, the water content of the tissue increased with increasing concentrations of K^+ in the medium, both during leaching and during incubation; the increase was rather small up to 40 mM-K^+. While the content of

K^+ increased, the content of Na^+ fell. This result corresponds to experience with muscle (BOYLE and CONWAY, 1941) and with kidney cortex slices (CORT and KLEINZELLER, 1957). It would appear that the effect of K^+ on the hydration of brain

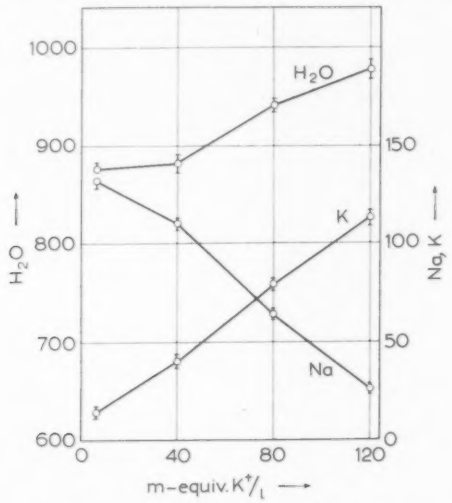

FIG. 2.—The effect of varying concentrations of K^+ on water and cation contents in rabbit brain cortex slices during leaching. Slices leached in 0·154 M-NaCl at 0° for 2 hr. Distribution marks are ±S.E., each point having been calculated from six estimations, made in duplicate. Results expressed in ml or m-equiv./100 g dry solids.

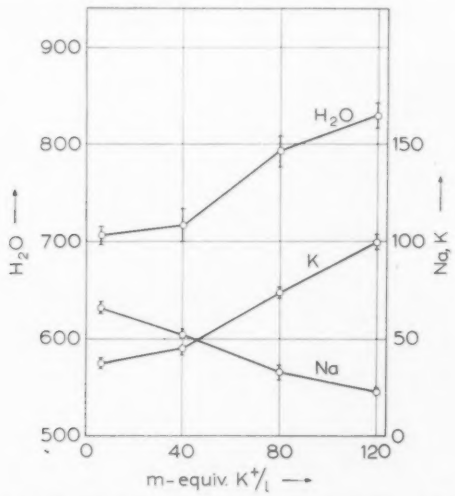

FIG. 3.—The effect of varying concentrations of K^+ on water and cation contents in rabbit brain cortex slices during incubation. Slices incubated 30 min at 30° in phosphate saline, containing 0·4% glucose; O_2 as gaseous phase. Distribution marks are ±S.E., each point having been calculated from eight estimations, made in duplicate. Results expressed in ml or m-equiv./100 g dry solids.

tissue follows the mechanism shown for muscle tissue by BOYLE and CONWAY. While PAPPIUS and ELLIOTT found practically the same degree of hydration of brain cortex slices after incubation at 38° for 1 hr in media containing 40 and 100 mM-K^+, in experiments reported here an increase of hydration between these concentrations

was observed. This discrepancy may possibly be explained by differences in the experimental animals used (rat and rabbit) and in the experimental conditions. The considerable amount of material available made it possible in our experiments to measure directly the degree of hydration at various concentrations of K^+ in the medium.

(2) *The effect of glutamic and aspartic acids on glycogen synthesis and lactic acid formation in brain cortex slices*

Sodium glutamate (0·01 M), when present in the incubation medium, markedly decreased the synthesis of glycogen, as shown in Table 1. A somewhat smaller

TABLE 1.—THE EFFECT OF Na GLUTAMATE AND Na ASPARTATE ON GLYCOGEN SYNTHESIS AND LACTIC ACID FORMATION IN BRAIN CORTEX SLICES

Amino acid added	Initial glycogen content	Glycogen content and lactic acid formed after incubation			
		Controls		Amino acid added	
		Glycogen	Lactic acid	Glycogen	Lactic acid
Na glutamate	211	612	9·6	401	25·3
Na glutamate	240	528	8·7	353	19·8
Na glutamate	256	526	5·3	287	17·9
Na glutamate	265	596	8·2	395	25·6
Na aspartate	318	539	8·8	441	12·8
Na aspartate	232	516	6·8	434	10·1
Na aspartate	269	542	7·2	438	12·2
Na aspartate	273	508	7·4	448	11·8

Slices incubated 3 hr at 37° in phosphate saline, containing 0·4% glucose, with or without 0·01 M-Na glutamate or 0·01 M-Na aspartate; Oxygen as gaseous phase. Glycogen expressed in μg glucose/g wet weight; lactic acid in mg/g wet weight.

effect was produced by the same concentration of Na aspartate both on glycogen synthesis and on lactic acid formation (Table 1).

It is known that adenosinetriphosphate (ATP) abolishes the effect of glutamic acid on anaerobic glycolysis (STERN *et al.*, 1949). As shown in Table 2, 2×10^{-3} M-ATP also partly abolished the inhibitory effect of glutamic acid on glycogen synthesis.

(3) *The effect of cholinesterase blocking agents on glycogen synthesis and transport of cations and water*

Compound 284 C 51 j, a selective inhibitor of true acetylcholinesterase (BAYLISS and TODRICK, 1956), at a concentration of 10^{-3} M, and 10^{-4} M-lysivane, a selective inhibitor of pseudocholinesterase, both markedly inhibited glycogen synthesis in brain cortex slices (Table 3). In some experiments not reported here, an inhibitory effect of compound 284 C 51 on glycogen synthesis was observed even at a concentration of 5×10^{-4} M.

TABLE 2.—THE EFFECT OF ATP ON GLYCOGEN SYNTHESIS IN BRAIN CORTEX SLICES IN THE PRESENCE OF GLUTAMATE

Initial glycogen content	Glycogen content and lactic acid formed after incubation			
	Na glutamate		Na glutamate + ATP	
	Glycogen	Lactic acid	Glycogen	Lactic acid
280	342	22·6	393	17·4
213	235	19·5	310	16·4
288	241	17·3	345	10·9
290	317	17·5	373	10·4

Slices incubated 3 hr at 37°, with O_2 as gaseous phase, in phosphate saline containing 0·4% glucose and 0·01 M-glutamate, with or without 0·002 M-ATP. Glycogen expressed in μg glucose/g wet weight, lactic acid in mg/g wet weight.

TABLE 3.—THE EFFECT OF CHOLINESTERASE INHIBITORS ON GLYCOGEN SYNTHESIS AND LACTIC ACID FORMATION IN BRAIN CORTEX SLICES

Added inhibitor	Initial glycogen content	Glycogen content and lactic acid formed after incubation			
		Controls		Inhibitor added	
		Glycogen	Lactic acid	Glycogen	Lactic acid
5×10^{-3} M-284 C 51 j	227	358	8·1	268	22·2
5×10^{-3} M-284 C 51 j	156	332	—	152	—
5×10^{-3} M-284 C 51 j	264	516	7·8	192	24·0
5×10^{-3} M-284 C 51 j	219	420	5·8	189	22·2
1×10^{-3} M-284 C 51 j	202	446	5·9	295	9·1
1×10^{-3} M-284 C 51 j	—	532	4·3	396	7·5
5×10^{-4} M-lysivane	337	565	5·0	305	8·2
5×10^{-4} M-lysivane	195	510	5·8	178	9·1
1×10^{-4} M-lysivane	290	558	—	450	—
1×10^{-4} M-lysivane	307	467	—	397	—

Slices incubated 3 hr at 37° in phosphate saline, containing 0·4% glucose, with or without inhibitor; O_2 as gaseous phase. Glycogen expressed in μg glucose/g wet weight, lactic acid in mg/g wet weight.

A decrease of glycogen synthesis in the presence of these inhibitors might be caused in a number of ways. The inhibitors might act (a) on enzyme systems directly involved in glycolysis and glycogen synthesis, or (b) by affecting the energy metabolism of the cell (i.e. the formation of high-energy bonds), or (c) by changing the intracellular concentration of K^+, since it is known that such substances affect cation transport

in brain cortex slices (STRICKLAND and THOMPSON, 1955; STRICKLAND, THOMPSON and WEBSTER, 1956). It is known that K^+ accumulates, at least in liver tissue, during glycogen synthesis (FENN, 1939). The mechanism of the observed inhibitory effect was therefore examined in more detail.

Of both inhibitors studied, only lysivane at a concentration of 10^{-3} M affected cellular permeability for the passive transport of sodium and potassium during leaching in isotonic NaCl; it also appeared to affect the normal course of active transport of both cations during incubation at a concentration as low as 5×10^{-4} M (Tables 4, 5, 6, 7). Neither of these inhibitors had any effect on the glycolytic activity of a MEYERHOF (1926) extract prepared from rabbit leg muscle. The view thus appears to be justified that neither lysivane nor compound 284 C 51 interfere directly with any of the enzyme equilibria of the glycolytic system.

However, as shown in Table 8, lysivane brought about a considerable increase in respiration of brain cortex slices, and also a marked decrease of anaerobic

TABLE 4.—THE EFFECT OF 1×10^{-3} M-LYSIVANE ON THE TRANSPORT OF CATIONS AND WATER IN BRAIN CORTEX SLICES DURING LEACHING

I			T_0 in NaCl			T_0 in NaCl + lysivane		
H_2O	Na^+	K^+	H_2O	Na^+	K^+	H_2O	Na^+	K^+
(ml)	(m-equiv.)		(ml)	(m-equiv.)		(ml)	(m-equiv.)	
482	48·8	36·1	809	99·7	5·8	840	111·6	4·4

Slices of guinea pig brain cortex leached at 0° for 2 hr in 0·154 M-NaCl, to which 0·1 volume of 0·1 M-phosphate buffer pH 7·4 was added. Average of four experiments. All values in ml or m-equiv./100 g dry solids.

TABLE 5.—THE EFFECT OF 1×10^{-3} M AND 5×10^{-4} M-LYSIVANE ADDED DURING INCUBATION ON THE TRANSPORT OF CATIONS AND WATER IN BRAIN CORTEX SLICES

Time interval	H_2O (ml)	Na^+ (m-equiv.)	K^+
I	491	36·1	34·2
T_0	822	96·3	8·1
T_{30}	772	81·2	23·1
T_{30} + 1×10^{-3} M-lysivane	817	92·8	11·8
T_0	945	116·0	4·8
T_{30}	803	96·2	20·6
T_{30} + 5×10^{-4} M-lysivane	814	102·4	18·0

Slices of guinea pig brain cortex leached at 0° for 2 hr in buffered 0·154 M-NaCl, then incubated in 3 ml phosphate saline containing 0·4% glucose for 30 min at 30°. All values averages of four experiments and expressed in ml or m-equiv./100 g dry solids.

TABLE 6.—THE EFFECT OF 5×10^{-3} M-284 C 51 j ON THE TRANSPORT OF CATIONS AND WATER IN BRAIN CORTEX SLICES DURING LEACHING

I			T_0 in NaCl			T_0 in NaCl + 284 C 51 j		
H_2O (ml)	Na^+ (m-equiv.)	K^+	H_2O (ml)	Na^+ (m-equiv.)	K^+	H_2O (ml)	Na^+ m(-equiv.)	K^+
495	36·4	34·3	913	123·0	7·6	892	119·9	8·2

Slices of guinea pig brain cortex leached at 0° for 2 hr in 0·154 M-NaCl, to which 0·1 volume of 0·1 M-phosphate buffer pH 7·4 was added. Average of four experiments. All values in ml or m-equiv./100 g dry solids.

TABLE 7.—THE EFFECT OF 5×10^{-3} M-284 C 51 j ADDED DURING INCUBATION ON THE TRANSPORT OF CATIONS AND WATER IN BRAIN CORTEX SLICES

Time interval	H_2O (ml)	Na^+ (m-equiv.)	K^+
I	528	41·8	32·2
T_0	823	106·1	8·3
T_{30}	807	78·5	21·8
$T_{30} + 5 \times 10^{-3}$ M-284 C 51 j	800	86·0	20·8

Slices of guinea pig brain cortex leached at 0° for 2 hr in buffered 0·154 M-NaCl, then incubated in 3 ml phosphate saline containing 0·4% glucose for 30 min at 30°. All values averages of four experiments and expressed in ml or m-equiv. per 100 g dry solids.

TABLE 8.—THE EFFECT OF CHOLINESTERASE INHIBITORS ON RESPIRATION AND ANAEROBIC GLYCOLYSIS IN BRAIN CORTEX SLICES

	Control	5×10^{-4} M-lysivane	5×10^{-3} M-284 C 51 j
Q_{O_2}	−10·1	−16·2	−9·5
RQ	1·09	1·3	—
$Q^{N_2}_{CO_2}$	17·0	7·6	—

Slices incubated 1·5 hr at 37°. Further experimental details see Methods.

glycolysis. Compound 284 C 51 had no comparable effect. Similar results have been obtained with kidney cortex slices (KLEINZELLER and CORT, 1957).

The results suggest that lysivane decreases glycogen synthesis by affecting the energy metabolism (i.e. oxidative phosphorylation) of brain cortex slices. Such a view would also explain the observed decrease of sodium extrusion from the cells, brought about by lysivane, since metabolic energy is required for active transport processes (see, for example, USSING, 1954). A similar conclusion was reached by

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STRICKLAND *et al.* (1956) with regard to the effect of some other cholinesterase inhibitors (di*iso*propylphosphofluoridate, eserine and compound Nu 1250) on the energy metabolism of brain cortex cells. These results give no evidence that pseudocholinesterase participates in the mechanism of cation transport across cellular membranes of brain cortex cells, since inhibitors of this enzyme inhibit at the same concentrations both cation transport and the Pasteur effect.

In order to find out whether the intracellular potassium concentration plays a part in the action of lysivane on glycogen synthesis in brain cortex slices, the effect of 40 mM-K^+ on glycogen synthesis in the presence of 5×10^{-4} M-lysivane was examined. As shown above, at this concentration of K^+, the content of K^+ in the slices is about equal to that in intact tissue, and glycogen synthesis is not impaired; on the other hand, 5×10^{-4} M lysivane markedly inhibits glycogen synthesis. As shown in Table 9, the presence of 40 mM-K^+ greatly decreased the inhibitory effect of lysivane.

TABLE 9.—THE EFFECT OF K^+ ON GLYCOGEN SYNTHESIS IN BRAIN CORTEX SLICES IN THE PRESENCE OF LYSIVANE

Glycogen content after incubation		
Controls	5×10^{-4} M-lysivane	40 m-equiv. K^+ + 5×10^{-4} M-lysivane
660	343	582
532	437	534
560	399	635
—	303	408
—	342	408
—	314	439
—	322	544

Slices incubated 3 hr at 37°; O_2 as gaseous phase. Glycogen expressed in μg glucose/g wet weight.

DISCUSSION

(1) *The effect of K^+ on the metabolism of brain cortex slices and the mechanism of its action.* The observation that increased concentrations of K^+ ions in the medium bring about a decrease in glycogen synthesis appears to agree with the known effect of K^+ on aerobic glycolysis (ASHFORD and DIXON, 1935; DICKENS and GREVILLE, 1935), in producing an increased breakdown of polysaccharides. It may be assumed that the same mechanism will apply in both cases.

Various views have been expressed concerning the mechanism of the action of increased K^+ concentrations on metabolic processes in brain cortex (ASHFORD and DIXON, 1935; DICKENS and GREVILLE, 1935; LIPSETT and CRESCITELLI, 1950; MCILWAIN, 1952; TSUKADA and TAKAGAKI, 1955). The experimental evidence obtained by a number of workers indicates that the energy metabolism of the cells is affected. An increased concentration of K^+ in the medium brings about a reversal of the Pasteur effect, a decrease of the level of phosphocreatine in the tissue (MCILWAIN,

1952), a decreased incorporation of inorganic phosphate into organic phosphate fractions (FINDLAY, MAGEE and ROSSITER, 1954); it also causes a decrease in glycogen synthesis and increase in hydration as reported above. This last effect may be due also to some extent to an increased breakdown of phosphocreatine and of some macromolecules within the cells (CONWAY, GEOGHEGAN and MCCORMACK, 1955), thus increasing the intracellular concentration of osmotically active substances. Recently, a decrease of ammonia formation with increasing concentrations of K^+ in the medium was observed in brain cortex slices (VRBA, FOLBERGR and KANTŮREK, 1957). Since ammonia formation in brain cortex slices is inhibited by such enzyme inhibitors as dinitrophenol, and other uncoupling agents of oxidative phosphorylation and inhibitors of oxidative processes (WEIL-MALHERBE and GREENE, 1955), it would appear that ammonia formation in brain is also associated with the energy metabolism of this tissue, and thus affected by K^+ ions.

Since brain cortex cells in the presence of oxygen, and a substrate, establish and maintain a concentration gradient of K^+ against the medium, increased concentrations of K^+ in the medium can have little effect on its intracellular concentration. Nevertheless, considerable changes of the potential difference across the cellular membrane may occur. The summary presented above indicates that high concentrations of K^+ in the extracellular fluid inhibit those metabolic processes which require high-energy bonds. Thus the suggestion may be made that K^+ in some way activates the breakdown of high-energy phosphate bonds directly at (or near) the cell membrane, so that fewer high-energy phosphate bonds are available for such endothermic processes as glycogen synthesis and water transport. Such a view might be supported by the recent observation of SKOU (1957) that brain ATPase is activated under certain conditions by potassium ions, and also by the finding of HEALD (1954) that incubation of brain slices in a medium containing 90 mM-K^+ brings about a complete breakdown of creatinephosphate within 60 sec. In the giant squid axon ATPase is localized in the sheath (LIBET, 1948); if this type of cell is analogous to those of brain cortex, the enzyme may be located at or near the cell membrane.

(2) *The effect of glutamic and aspartic acids on glycogen synthesis.* Glycogen synthesis is also inhibited by glutamic acid, as shown independently by one of us (RYBOVÁ, 1955) and by MCILWAIN and TRESIZE (1956). ACS *et al.* (1953) found that in the presence of glutamic acid a considerable amount of ATP is utilized in the synthesis of glutamine. Furthermore, glutamic acid decreases the incorporation of ^{32}P into organic phosphate fractions (ENGELGARDT and LISSOVSKAIA, 1953; FINDLAY *et al.*, 1954). It would appear that enzyme systems involved in both glycogen and glutamine synthesis compete with each other for high-energy phosphate bonds, and thus less ~P is available for glycogen synthesis if glutamate is present. Such an interpretation is supported by the observation, reported above, that addition of ATP to the medium partly abolishes the effect of glutamic acid, although it is difficult to see how added ATP acts, since the cell membrane is considered to be impermeable to this substance. The view proposed above on the localization of ATPase might help to explain these observations.

The question arises of the source of the ammonia required for glutamine synthesis, since no ammonium ions were added to the medium in our experiments. It appears likely that ammonia is formed as an intermediate by the deamination of glutamic acid, and then reacts with another molecule of glutamic acid to form glutamine, as

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suggested by FINDLAY *et al.* (1954). As another source of ammonia, protein-bound amide bonds may also be considered (VRBA, 1955).

It was found that aspartic acid markedly decreased glycogen synthesis. ACS *et al.* (1953) also found that apart from glutamic acid, only aspartic acid brought about a decrease of ATP in brain cortex slices. The conclusion of these authors that aspartic acid forms glutamic acid by transamination with α-ketoglutaric acid, may also be considered as an explanation of the effect of aspartate on glycogen synthesis in brain cortex slices.

(3) *The effect of cholinesterase blocking agents on glycogen synthesis and cation transport.* Both compounds with anticholinesterase activity, lysivane and compound 284 C 51, decreased glycogen synthesis in brain cortex slices. Lysivane, which was reported to inhibit selectively 90 per cent of pseudocholinesterase activity at a concentration of 5×10^{-5} M (BAYLISS and TODRICK, 1956), markedly decreases glycogen synthesis at a concentration of 1×10^{-4} M. At a concentration of 5×10^{-4} M this inhibitor decreased the transport of cations and water, and also appreciably increased the Q_{O_2} and RQ; it also decreased the $Q^{N_2}_{CO_2}$ in both brain cortex slices (Table 8) and kidney cortex slices (KLEINZELLER and CORT, 1957). It is known that some other cholinesterase inhibitors also inhibit sodium extrusion from cells during incubation *in vitro* (see USSING, 1954). STRICKLAND *et al.* (1955, 1956) have shown that di*iso*propylphosphofluoridate, Nu 1250 and eserine affected both cation transport and the energy metabolism of brain preparations at concentrations at least one order higher than required for complete inhibition of cholinesterase activity. This observation suggested that the inhibition of cation transport is due to interference by the inhibitors with the energy metabolism of the cell rather than to a direct effect on transport processes. The results reported above would appear to support the conclusions of STRICKLAND *et al.* (1955, 1956).

On the other hand, the observation that an increased concentration of K^+ in the medium abolished the effect of lysivane on glycogen synthesis appears to suggest that glycogen synthesis is impaired in the presence of the inhibitor as a consequence of loss of K^+ from the cells. Thus a possible participation of pseudocholinesterase in the transport of cations in brain cortex cells cannot yet be excluded.

Compound 284 C 51 j, which quantitatively inhibits true acetylcholincholinesterase at a concentration of 3×10^{-5} M, also decreases glycogen synthesis in brain cortex slices at concentrations of 1×10^{-3} M and 5×10^{-4} M, without affecting cation transport, oxygen uptake, or glycolysis by Meyerhof's muscle extract, even at concentrations as high as 5×10^{-3} M. For the time being no suggestion can be offered for the mechanism by which this substance affects glycogen synthesis.

SUMMARY

(1) The effect of varying concentrations of K^+ on glycogen synthesis and on the cation and water consent of brain cortex slices was examined. It was found that:

(a) glycogen synthesis was decreased by concentrations of K^+ higher than 40 mM. Within the concentration range of 6·4 to 40 mM-K^+, no effect of K^+ on glycogen synthesis was observed;

(b) increasing concentrations of K^+ in the medium brought about an increase of water and K^+ content, and a decrease of Na^+ content in the slices, both on leaching at 0°C and on incubation at 30°C.

(2) Sodium glutamate (0·01 M) and aspartate (0·01 M) decreased glycogen synthesis and increased lactic acid formation in brain cortex slices. The effect of glutamate on glycogen synthesis was abolished by 2×10^{-3} M-adenosinetriphosphate.

(3) The effect of lysivane, a selective inhibitor of pseudocholinesterase, and of compound 284 C 51 j, a selective inhibitor of true acetylcholinesterase, on glycogen synthesis, cation transport and the Pasteur effect in brain cortex slices was examined. It was found that:

(a) lysivane (10^{-4} M) decreased glycogen synthesis, increased Q_{O_2} $Q^{O_2}_{CO_2}$ and RQ, somewhat decreased $Q^{N_2}_{CO_2}$, and inhibited (5×10^{-4} M) Na^+ extrusion and reaccumulation of K^+. Lysivane had no effect on a glycolytic system.

(b) the effect of lysivane on glycogen synthesis was abolished by raising the concentration of K^+ to 40 mM.

(c) compound 284 C 51 j (5×10^{-4} M) inhibited glycogen synthesis in brain cortex slices, without affecting respiration, cation transport or glycolysis.

(4) The mechanism of action of K^+, glutamate and cholinesterase inhibitors on the metabolism of brain cortex slices is discussed.

Acknowledgements—The authors are greatly indebted to J. KVASNICKOVÁ for invaluable technical assistance. A gift of lysivane from May and Baker, Ltd., and of compound 284 C 51 from Dr. F. C. COPP, Burroughs Wellcome Research Laboratories, is gratefully acknowledged.

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Journal of Neurochemistry, 1957, Vol. 2, pp. 58-70, Pergamon Press Ltd., London

THE SIMULTANEOUS FLUORIMETRIC DETERMINATION OF ADRENALINE AND NORADRENALINE IN PLASMA—I*

THE FLUORESCENCE CHARACTERISTICS OF ADRENOLUTINE AND NORADRENOLUTINE AND THEIR SIMULTANEOUS DETERMINATION IN MIXTURES

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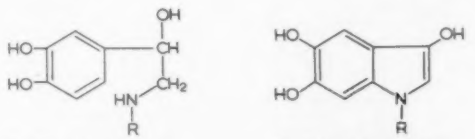
(*Received* 23 *June* 1957)

INTRODUCTION

THE fluorescence of alkaline solutions of adrenaline was first reported by PAGET (1930). Subsequently, GADDUM and SCHILD (1934) noted a similar fluorescence, but of much weaker intensity, for noradrenaline. LUND (1949*a*,*b*) isolated and identified the fluorescent product of adrenaline as the 5:6-dihydroxyindoxyl, which he termed adrenolutine (Fig. 1). By analogy, the fluorescent product derived from noradrenaline was assumed to be the corresponding noradrenolutine (LUND, 1950).



R = CH_3 = Adrenaline
R = H = Noradrenaline

R = CH_3 = Adrenolutine
R = H = Noradrenolutine

FIG. 1.—Structures of adrenaline, noradrenaline and corresponding lutines.

LUND (1950) further reported a differential analysis for adrenaline and noradrenaline. It was based upon the pH-controlled oxidation of the catechol amines, followed by tautomerization to the lutines by alkali. L-Ascorbic acid added to the alkali stabilized the fluorescence.

HELLER *et al.* (1950) reported that the maximum fluorescence of alkaline adrenaline solutions occurred at 540 mμ, while that for noradrenaline occurred at about 520 mμ. Differences in excitation (activation of fluorescence) between the ascorbic acid-stabilized lutines were observed by GOLDENBERG *et al.* (1954). The ratios of fluorescence obtained by excitations with the 365 and 436 mμ lines of the mercury lamp,

* This work was supported by a grant from the U.S. Public Health Service, National Institutes of Health (Research Grant H-1045 (C5)).

with their particular instrument and filters, were 0·7 for adrenolutine and 2·0 for noradrenolutine.

We were interested in utilizing the fluorescence of the lutines for the determination of adrenaline and noradrenaline in plasma extracts. However, we were not able to reproduce LUND's preferential oxidation method satisfactorily. We therefore decided to investigate the possibility of utilizing differences in the fluorescence characteristics of the lutines as a basis for differential analysis. In this present investigation, the fluorescence characteristics of the lutines have been examined in detail with a filter fluorimeter and two spectrophotofluorimeters. The data have been applied to the development of a new fluorimetric method for the simultaneous determination of adrenaline and noradrenaline in plasma. An outline of the procedure follows:

(1) Adrenaline and noradrenaline are adsorbed on to aluminum oxide and subsequently eluted with acetic acid.

(2) The samples are oxidized with manganese dioxide at pH 6·5–7·5 and then split into two equal portions.

(3) The fluorescent adrenolutine and noradrenolutine are formed by tautomerization with alkali. In one portion, the presence of L-ascorbic acid in the alkali stabilizes the lutines; in the other portion (blank), the alkaline lutines are allowed to decompose to non-fluorescent compounds.

(4) The samples are fluorimetrically analysed with two sets of filters. Each filter set yields a different ratio of fluorimetric readings from adrenolutine and noradrenolutine.

(5) The quantities of adrenaline and noradrenaline are calculated by solving a set of simultaneous equations.

This present paper deals only with the fluorescence characteristics of adrenolutine and noradrenolutine and methods for their simultaneous fluorimetric determination. The second paper of this series (COHEN and GOLDENBERG, 1957) describes the application to plasma analyses.

EXPERIMENTAL

Reagents. L-Epinephrine (adrenaline) bitartrate and L-norepinephrine (noradrenaline) bitartrate monohydrate were obtained from Winthrop Stearns, Inc., New York. The adrenaline complex was recrystallized from methyl alcohol-petroleum ether.

L-Ascorbic acid (reagent grade, Fisher Scientific Co., New York) was thrice recrystallized from distilled water. This resulted in the removal of some extraneous fluorescence.

Manganese dioxide (c.p., assay 85 per cent, Matheson, Coleman and Bell, Norwood, Ohio) was shaken with several litre portions of N-sodium hydroxide and the supernatant liquid decanted. It was then washed in a similar manner with distilled water until neutral pH (pH 6) had been reached, filtered on a Buchner funnel, and air dried. The material so obtained contributed no detectable fluorescence to the samples and packed well on centrifugation.

Reagent grade sodium hydroxide, sodium acetate and acetic acid were utilized without further purification.

Preparation of samples. Stock solutions of L-adrenaline bitartrate and L-noradrenaline bitartrate monohydrate were prepared in 0·01 N-hydrochloric acid or in distilled water, at concentrations corresponding to 1 mg catechol amine per ml. For convenience in handling, stock dilutions of 0·5 or 1·0 μg adrenaline or noradrenaline per ml distilled water were prepared.

Standard solutions for fluorescence measurements were prepared in calibrated 15 ml ground glass stoppered centrifuge tubes, in the presence of 1 ml M-acetate buffer pH 6·5, and sufficient distilled water to make the volume 8·0 ml. Samples were oxidized to adrenochrome and noradrenochrome by the addition of 10–20 mg manganese dioxide from the tip of a spatula, followed by repeated inversion of the tubes for 1 min. After centrifugation (700 G) for 1 or 2 min., the supernatant liquid was

decanted into clean test tubes (2 cm × 12 cm). The fluorescent lutines were formed by the addition of 1 ml fresh alkaline ascorbic acid solution, prepared by mixing 1 ml freshly prepared aqueous L-ascorbic acid (100 mg per 10 ml) with 5 ml 5 N-sodium hydroxide. Samples were mixed by gently tapping the side of each tube with the forefinger. Adrenolutine formed immediately, whereas noradrenolutine formed over a 4–6 min. period. At this stage, no more than a 5 per cent decomposition of the lutines could be detected in a 45 min. period, in samples which were not subjected to further agitation.

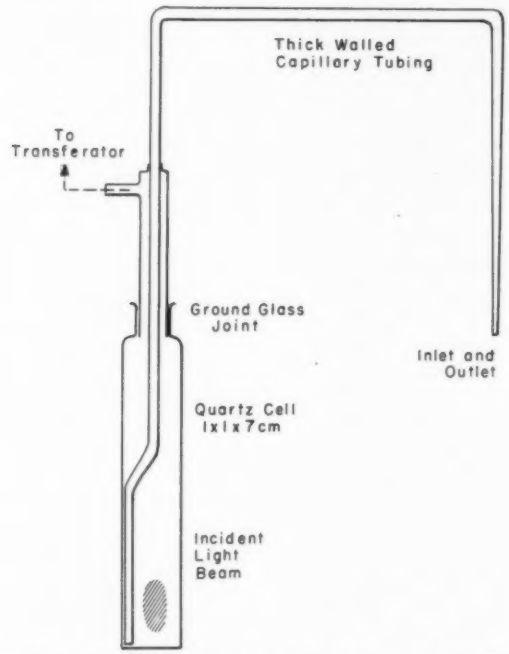


FIG. 2.—Quartz fluorimeter cell and attachments. Schematic, side view.

A routine reagent blank was prepared with each set of standard solutions. It consisted of 1 ml M-acetate buffer pH 6·5, and 7 ml distilled water; it was shaken with manganese dioxide and made alkaline as described above.

Fluorescence measurements. An experimental model of the Farrand spectrophotofluorimeter (Farrand Optical Co., Inc., Bronx, N.Y.), and a standard Aminco-Bowman spectrophotofluorimeter (American Instrument Co., Silver Springs, Maryland) were utilized in this investigation. The filter fluorimeter employed was a Farrand fluorimeter, Model A. The instrument had been modified by the manufacturer to include two sets of coarse and fine sensitivity switches, which could be alternately activated.

The data of Tables 1 and 2 were obtained in the conventional manner, utilizing the standard Farrand cuvette holder, matched cuvettes, and one of the two sets of sensitivity switches. However, when the highest sensitivity was required, as in Fig. 7 or Table 3, the Farrand cuvette holder was removed and a brass compartment substituted. This brass compartment was designed to fit snugly in place and to hold rigidly a single square quartz cell (1 × 1 × 7 cm) of weak natural fluorescence, which in turn was equipped with a ground glass joint (Pyrocell Mfg. Co., New York, N.Y.).

Samples for fluorimetric analysis were forced in or out of the cell by means of an automatic transferator (Gilson Medical Electronics, Madison, Wisconsin). Entry to, and exit from, the cell was afforded by capillary tubing passing through the ground glass joint and extending along one corner of the cell, out of the path of the light beam, to within 0·1 cm of the bottom (Fig. 2). The

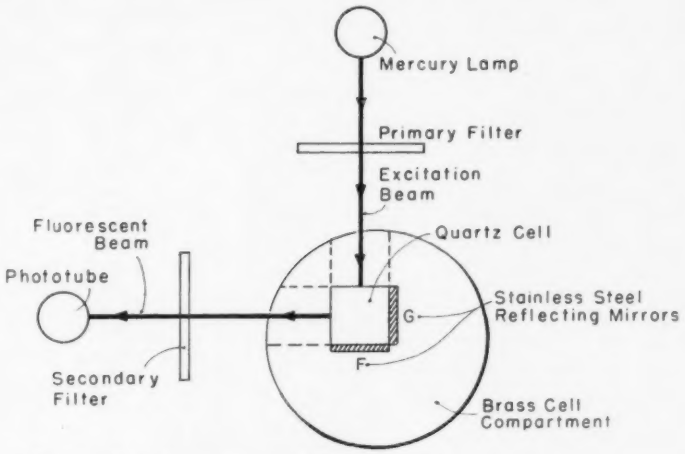


FIG. 3.—Optical arrangement of fluorimeter. Schematic, top view.

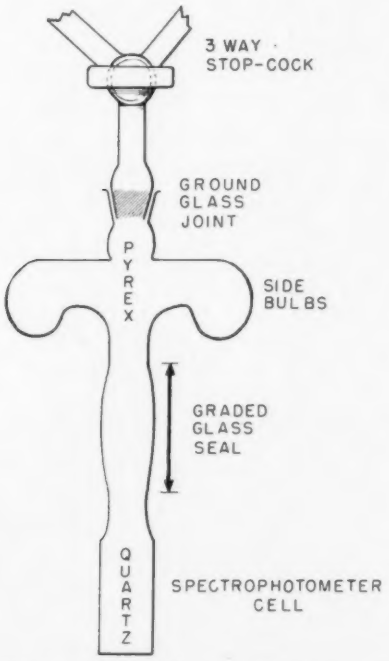


FIG. 4.—Spectrophotometer cell used for *in vacuo* optical density measurements. Schematic, side view. (Courtesy of Professor CHARLES R. DAWSON, Department of Chemistry, Columbia University.)

native fluorescence of polyethylene tubing precluded its substitution for the glass capillary tubing. The transferator functioned by alternately creating a vacuum or exerting pressure. The use of a single cell for all fluorimetric readings eliminated any variations due to differences in cuvettes.

Increased fluorimetric sensitivity was obtained by employing two stainless steel mirrors (Fig. 3), outside, and adjacent to, the quartz cell. One of these *F* was located at right angles to the incident (activating) light beam, and reflected incident light back through the solution. The other *G* was located at right angles to the first mirror. Fluorescent radiation incident upon mirror *G* was reflected back to the phototube.

Two sets of filter combinations were used to illustrate the general method for simultaneous determinations of adrenaline and noradrenaline (see Results). The first set (A) consisted of a primary (activation) combination of Corning 3060, Farrand 405 mμ interference filter, and Corning 5970 (listed in order of placement adjacent to the mercury lamp), used in conjunction with an Ilford-Bright 623 in the secondary (fluorescence) position. The second set (B) consisted of a primary combination of Corning 3389, Farrand 436 mμ interference filter, and a one-half standard thickness Corning 5113 (listed in order of placement adjacent to the mercury arc lamp), used in conjunction with a Corning 3486 in the secondary position.

The fluorimeter cell was first filled with an oxidized and tautomerized adrenaline sample (0·05 μg), and the galvanometer standardized at 100 deflection units for one set of primary and secondary filters. The second set of filters was then substituted and the instrument similarly standardized with the alternate set of sensitivity switches. Diaphragm no. 4 was employed in both instances. The fluorescence of the remaining noradrenaline standard (0·05 μg), reagent blank, and remaining samples were then read immediately and successively by alternately changing filters and activating the appropriate set of sensitivity switches. Between samples, the fluorimeter cell was rinsed with distilled water several times.

Absorption spectra of the lutines. The absorption spectra of the lutines were recorded in the absence of ascorbic acid, whose strong ultraviolet absorption would have interfered with optical density measurements. Deoxygenation of the oxidized adrenaline and noradrenaline samples prevented decomposition (oxidation) of the fluorescent lutines in the presence of alkali.

Solutions of 50 μg adrenaline or noradrenaline in 0·5 ml M-acetate buffer, pH 6·5, and sufficient distilled water to make the final volume 4·0 ml, were oxidized in the usual manner with 50 mg manganese dioxide. The pink adrenochrome or noradrenochrome solution present after centrifugation of the manganese dioxide was transferred to a quartz and glass container. This container (Fig. 4) consisted of a 1 cm^2 quartz spectrophotometer cell, attached by means of a graded glass seal, to a Pyrex ground glass joint carrying a three-way stopcock. Two side bulbs of 2 ml capacity each were located between the three-way stopcock and the quartz cell. The adrenochrome or noradrenochrome solution was placed in the lower (quartz) portion of the cell, and 0·2 ml of 12·5 N-sodium hydroxide in one side bulb. Evacuation (deoxygenation) was achieved in several minutes with the aid of a high vacuum pump. During deoxygenation the container was held at a 20–30° angle to the ground. In this manner, violent bubbling (degassing) and premature mixing of the samples were prevented. The samples were mixed with sodium hydroxide *in vacuo* and allowed to stand for 10 min to ensure complete conversion to the lutines.

Spectra were recorded on a Beckman DU spectrophotometer. Aluminium foil was employed to keep the spectrophotometer cell compartment light-tight. The lutines were completely stable under these conditions as evidenced by unchanging optical density readings at several wavelengths. A similarly prepared reagent blank was utilized to obtain optical density corrections for the absorption spectra of the lutines.

RESULTS

Absorption spectra. The absorption spectra of adrenolutine and noradrenolutine are presented in Fig. 5. Absorption maxima occurred at 275, 325 and 408 mμ for adrenolutine and at 270, 325 and 396 mμ for noradrenolutine. The strong ultraviolet absorption of L-ascorbic acid precluded its use as a stabilizer of the lutines during ultraviolet absorption measurements. Stabilization of the lutines, i.e. the prevention of decomposition via oxidation, was attained in this instance by careful deoxygenation of the solutions prior to lutine formation.

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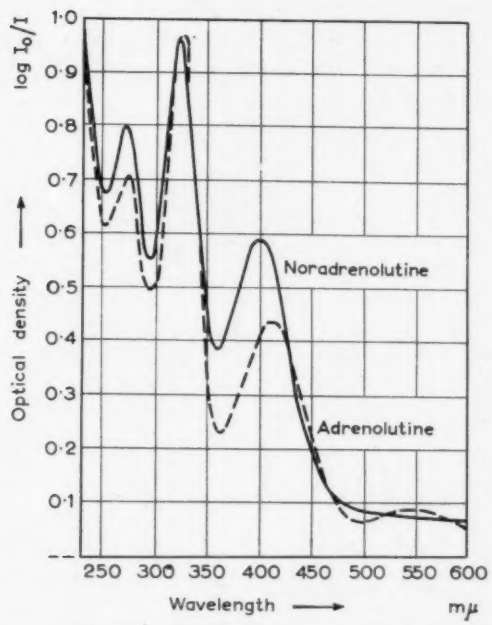

FIG. 5.—Absorption spectra of alkaline solutions of adrenolutine and noradrenolutine (12 μg per ml).

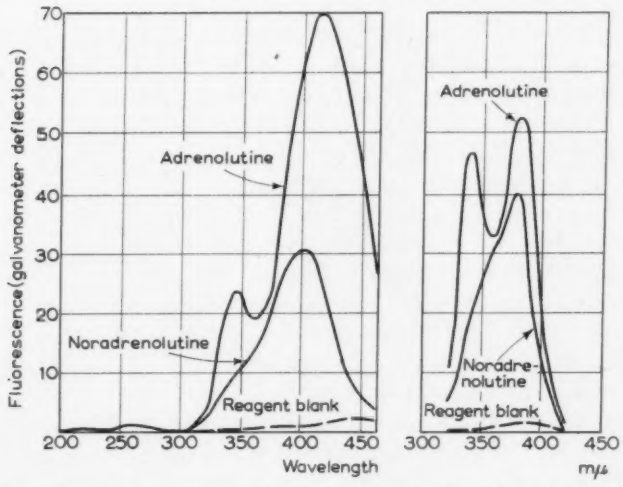

FIG. 6.—Excitation spectra of adrenolutine and noradrenolutine (0·04 μg per ml). Fluorescence measured at 515 mμ with Farrand spectrophotofluorimeter (*right*) and Aminco-Bowman spectrophoto fluorimeter (*left*).

Spectrophotofluorimeter data. In Fig. 6, the fluorescence observed at 515 mμ for adrenolutine and noradrenolutine have been plotted as a function of excitation (activation) wavelength. The excitation spectra obtained on both the Farrand and Aminco-Bowman spectrophotofluorimeters have been presented. L-Ascorbic acid was utilized for stabilization of the lutines.

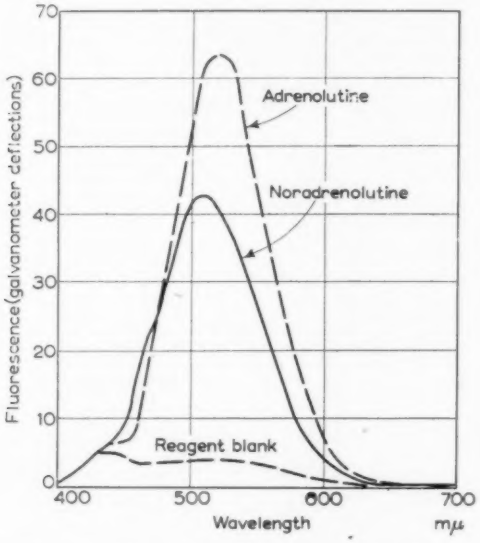

FIG. 7.—Fluorescence spectra of adrenolutine and noradrenolutine (0·04 μg per ml). Aminco-Bowman spectrophotofluorimeter and 380 mμ excitation.

Adrenolutine exhibited stronger fluorescence than noradrenolutine for excitations near 340 mμ, and also in the 390–410 mμ region. On the other hand, roughly equal intensity of fluorescence was observed in the 360–375 mμ region. No fluorescence was detectable for excitations below 300 mμ.

Maximal fluorescence was observed for excitations which corresponded closely with the absorption maxima exhibited by alkaline solutions of the lutines (Fig. 5). Furthermore, the adrenolutine excitation maximum (400 mμ region) was observed at somewhat higher wavelengths than that of noradrenolutine. This paralleled the corresponding locations of the absorption maxima.

Fig. 7 is a graphical presentation of the fluorescence observed for adrenolutine and noradrenolutine on the Aminco-Bowman instrument. A 380 mμ excitation was utilized. Similar fluorescence spectra were obtained on the Farrand spectrophotofluorimeter. Maximal fluorescence occurred at 510–515 mμ for noradrenolutine and 520–525 mμ for adrenolutine, on both spectrophotofluorimeters. Data recorded for other excitation wavelengths indicated that the wavelengths of maximal fluorescence were independent of excitation wavelength.

Filter fluorimeter data. In Tables 1 and 2, sample data are presented for selected filter combinations. The spectral regions isolated by the secondary filters are also indicated. For each set of filter combinations, the instrument was adjusted to 100 galvanometer deflection units for the lutine giving the strongest reading, and then the

TABLE 1.—FLUORESCENCE EMISSION CHARACTERISTICS OF ADRENOLUTINE AND NORADRENOLUTINE (0·02 μg/ml)

Farrand fluorimeter Model A and 365 mμ excitation

Secondary filter	Spectral area isolated	Galvanometer deflections			Ratio adr./noradr.
		Adr.	Noradr.	Blank	
1. Corning 3389	420–800 mμ	93·5	100·0	17·0	0·92
2. Corning 3385	480–800 mμ	100·0	100·0	11·0	1·00
3. Corning 3486	530–800 mμ	100·0	82·0	6·0	1·24
4. Corning 2418	605–800 mμ	100·0	66·0	10·0	1·61
5. Corning 3389 + 4308	420–600 mμ (480 mμ peak)	81·0	100·0	19·5	0·76
6. Corning 3385 + 4308	480–600 mμ (500 mμ peak)	88·0	100·0	11·5	0·86
7. Ilford-Bright 623	450–540 mμ (490 mμ peak)	83·0	100·0	20·0	0·79
8. Farrand Interference Filter	470 mμ peak	66·0	100·0	40·0	0·43
9. Farrand Interference Filter	490 mμ peak	64·0	100·0	13·0	0·59
10. Farrand Interference Filter	509 mμ peak	81·0	100·0	13·5	0·78
11. Farrand Interference Filter	530 mμ peak	100·0	100·0	19·0	1·00

TABLE 2.—EXCITATION CHARACTERISTICS OF ADRENOLUTINE AND NORADRENOLUTINE (0·02 μg/ml)

Farrand fluorimeter Model A

Excitation wavelength	Secondary filter	Spectral area isolated	Galvanometer deflections			Ratio adr./noradr.
			Adr.	Noradr.	Blank	
1. 365 mμ	Ilford-Bright 623	450–540 mμ peak at 490 mμ	83·0	100·0	20·0	0·79
2. 405 mμ			100·0	90·5	6·0	1·11
3. 436 mμ			100·0	35·0	11·0	3·7
4. 365 mμ	Corning 3486	530–800 mμ	100·0	82·0	6·0	1·24
5. 405 mμ			100·0	60·5	5·5	1·72
6. 436 mμ			100·0	20·5	4·0	5·8

alternate lutine and reagent blank were compared. Lutine concentrations of 0·02 μg per ml were employed.

Table 1 demonstrates more clearly than does the spectrophotofluorimeter the differences in the fluorescence spectra of the lutines. Data are presented for a 365 mμ excitation. The fluorescence of noradrenolutine relative to adrenolutine (corrected for reagent blank) was greatest in the spectral regions below 500 mμ, whereas adrenolutine exhibited its strongest relative fluorescence at longer wavelengths. Similar results were obtained with excitations at 405 or 436 mμ.

Table 2 demonstrates the variations obtained for excitations of 365, 405 and 436 mμ, as measured with two different secondary filters. The strongest relative excitation of noradrenolutine (compared to adrenolutine) occurred at 365 mμ, and the weakest at 436 mμ. This is in accord with the spectrophotofluorimetrically recorded excitation spectra.

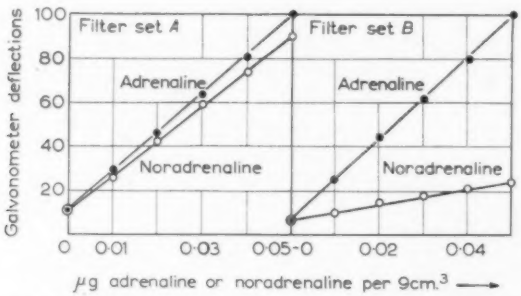

FIG. 8.—Standard fluorescence curves for adrenaline and noradrenaline. Samples converted to corresponding lutines. Filter set A consisted of a 405 mμ excitation and an Ilford-Bright 623 secondary filter. Filter set B consisted of a 436 mμ excitation and a Corning 3486 secondary filter.

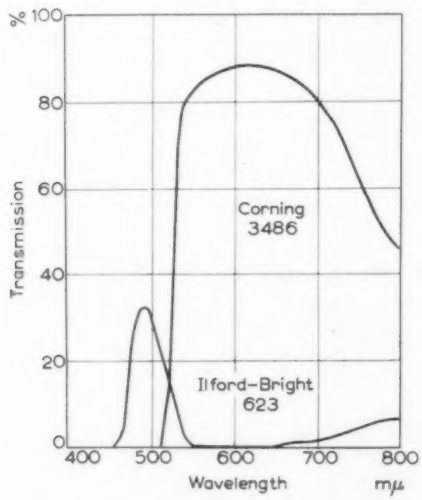

FIG. 9.—Transmission curves of Ilford-Bright 623 and Corning 3486 secondary filters.

Analytical method. The simultaneous determination of the lutines was accomplished on the Farrand filter fluorimeter by choosing two sets of primary (excitation) and secondary (fluorescence) filters, each giving different relative weights to the fluorescence of adrenolutine and noradrenolutine. Typical data for one such set of filter combinations are presented in Fig. 8. The fluorescence of the lutines was found to be additive, and furthermore no deviations from linearity could be detected in the concentration ranges employed (through 0·05 μg per 9 ml final volume). Duplicate

samples agreed to within 1 or 2 galvanometer deflection units. Similar results on the linearity of fluorescence had been reported previously for higher concentrations of the lutines and other filter combinations (LUND, 1950; GOLDENBERG *et al.*, 1954). The transmission curves of the secondary filters employed are presented in Fig. 9.

Typical analyses of single samples of mixed standards are presented in Table 3. The quantities of adrenaline and noradrenaline present before conversion to the lutines were calculated by substitution in the following set of simultaneous equations:

$$A = yN_a + xE_a \tag{1}$$

$$B = yN_b + xE_b \tag{2}$$

In these equations, x and y were the amounts of adrenaline and noradrenaline, respectively; A and B were the fluorimetric readings (corrected for the fluorescence of the reagents) for filter sets A and B, respectively; and E_a, E_b, N_a, and N_b were the values for the fluorescence per μg on filter sets A and B for adrenaline and noradrenaline, respectively.

Substitution of equation (1) into equation (2) yielded:

$$y = \frac{(AE_b/E_a) - B}{(N_aE_b/E_a) - N_b} \tag{3}$$

which was solved for y (amount of noradrenaline). The numerical value of x (amount of adrenaline) was subsequently determined by solution of equation (2) rearranged to form:

$$x = \frac{B - yN_b}{E_b} \tag{4}$$

Numerical values of E_a, E_b, N_a and N_b were obtained from fresh standards of adrenolutine and noradrenolutine, corrected for the fluorescence of the reagents.

TABLE 3.—SAMPLE SIMULTANEOUS ANALYSES OF ADRENALINE AND NORADRENALINE
(Samples converted to lutines in 9 ml standard volume for fluorimetric determinations)

Farrand filter fluorimeter Model A and filter sets A and B

Amount added ($\mu g \times 10^{-3}$)		Amount found ($\mu g \times 10^{-3}$)	
Adrenaline	Noradrenaline	Adrenaline	Noradrenaline
30·0	15·0	29·1	14·1
25·0	25·0	24·1	26·4
20·0	10·0	19·7	10·6
15·0	20·0	16·0	19·4
10·0	10·0	10·4	10·4
10·0	25·0	9·8	27·3
5·0	40·0	4·5	42·0
5·0	5·0	5·6	5·2

In the application of the above procedure to situations in which fluorescent contaminants were present, other than those in the reagents, e.g. plasma extracts (COHEN and GOLDENBERG, 1957), a blank was required to distinguish the fluorescence of the lutines from that of the contaminants. The sodium hydroxide blank employed by LUND in which decomposition of the lutines was allowed to occur (ascorbic acid stabilization omitted) in an aliquot portion of the extract, was found to be directly applicable.

DISCUSSION

The spectrophotofluorimeter is a relatively recently developed instrument (BOWMAN *et al.*, 1955), which provides greater spectral versatility than its precursor, the filter fluorimeter. A xenon lamp provides a continuous spectrum of visible and ultraviolet radiation from which a monochromator selects any desired wavelength for activation of fluorescence. A second monochromator permits selectivity in the analysis of the resultant fluorescence. The filter fluorimeter on the other hand, has only a limited number of excitation wavelengths available from its mercury lamp. Combinations of filters are employed for separation of the excitation wavelengths (primary filters) and for isolation of the spectral regions in the resultant fluorescence (secondary filters).

Marked differences between the adrenolutine and noradrenolutine excitation characteristics were detected both with the filter fluorimeter and the spectrophotofluorimeter. With the filter fluorimeter, excitation with the 365 mμ line of mercury elicited stronger fluorescence from noradrenolutine (relative to adrenolutine) than either 405 or 436 mμ excitations (Table 2). Similarly, the spectrophotofluorimetrically observed fluorescence (Fig. 6) was strongest for noradrenolutine (relative to adrenolutine) for excitations in the 360–375 mμ region, and much weaker elsewhere, particularly near 340 mμ and in the 390–410 mμ region.

The shapes of the spectrophotofluorimetrically recorded spectra were influenced by the optical characteristics of the instruments. The observed intensities of fluorescence were dependent upon the activating radiation intensities, and were therefore influenced by such factors as the energy distributions of the xenon lamp radiations and the light transmission efficiencies of the optical systems. The differences observed between the two spectrophotofluorimeters (Fig. 6) may be accounted for in terms of such factors.

Despite distortion of the spectrophotofluorimetrically recorded spectra there appeared to be close agreement between the absorption maxima of the lutines (Fig. 5) and the corresponding excitation maxima. This was anticipated in view of the dependence of the intensity of fluorescence upon the extent of absorption of activating radiation.

In addition to excitation differences, differences in the fluorescence emission spectra of the lutines were also detected both with the spectrophotofluorimeter and the filter fluorimeter. Again, it should be noted that the shapes of the spectrophotofluorimetrically recorded spectra (Fig. 7) were influenced by instrument factors. In this instance, the sensitivity characteristics of the phototubes and the light transmission characteristics of the optical systems were involved. In view of these factors, the duplication of recorded fluorescence maxima by both spectrophotofluorimeters appears to be fortuitous.

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The small differences in fluorescence spectra indicated by the spectrophotofluorimeter (Fig. 7) were demonstrated much more clearly with the filter fluorimeter (Table 1). Thus, with the appropriate choice of secondary filters, the apparent ratio of adrenolutine to noradrenolutine fluorescence varied from 0·43 (filter 8) to 1·61 (filter 4). The increase in ratio associated with the isolation of longer wavelengths (e.g. filters 1–4 or 8–11) indicated that the peak fluorescence of adrenolutine was located towards longer wavelengths than that of noradrenolutine. This was in agreement with the spectrophotofluorimetrically recorded spectra.

The simultaneous determination of adrenolutine and noradrenolutine was accomplished by utilizing excitation differences, fluorescence differences, or both. For instance, data obtained on the Farrand spectrophotofluorimeter by excitations at the 340 and 380 mμ peaks of adrenolutine (Fig. 6), could be substituted into equations (3) and (4) and the amounts of adrenaline and noradrenaline originally present calculated. Similarly, two excitation wavelengths could be chosen for use in conjunction with the filter fluorimeter, e.g. 365 and 436 mμ excitations with Corning 3486 secondary filter (filter combinations 4 and 6 of Table 2). Filter combinations 1 and 6 of Table 2 combined excitation and fluorescence differences with a resultant strong resolution of adrenolutine and noradrenolutine.

We have been employing the Farrand filter fluorimeter for routine assays. The filter combinations employed in Fig. 8 (combinations 2 and 6 of Table 2) have been found most convenient for the determination of adrenaline and noradrenaline in plasma extracts (COHEN and GOLDENBERG, 1957). The choice of filters was dependent upon the magnitude of the reagent blank fluorescence at the required sensitivity settings, and also upon the fluorescence characteristics of the contaminants present in the extracts. The relatively high sensitivities obtained in Fig. 8 were dependent upon the elimination of the round cuvette holder of the Farrand filter fluorimeter and its substitution by a rigidly held square quartz cell which was filled and emptied automatically from without, as described in the experimental section.

In general, any of the filter combinations listed in Tables 1 and 2, or other applicable combinations, may be employed for the simultaneous determination of the lutines. Reagent blanks may be minimized by choosing combinations of primary and secondary filters which are mutually exclusive to the transmission of light, i.e. secondary filters should not permit the transmission of any light passed by the primary filters. It should be borne in mind, however, that just as the spectrophotofluorimetrically-recorded spectra were a function of instrument factors, so are data obtained on the filter fluorimeter dependent upon the characteristics of the phototube, optical system and absorption of the filters. Therefore, some small differences in the qualitative picture herein described may be expected with other filter fluorimeters.

SUMMARY

A new method has been presented for the simultaneous fluorimetric determination of adrenaline and noradrenaline. Advantage was taken of the excitation and fluorescence emission differences between adrenolutine and noradrenolutine, the 5:6-dihydroxyindoxyls derived from adrenaline and noradrenaline.

Alkaline solutions of adrenolutine and noradrenolutine were studied fluorimetrically with a filter fluorimeter, and with both the Farrand and Aminco-Bowman spectrophotofluorimeters. The fluorescence emission spectrum of adrenolutine was

located towards longer wavelengths than that of noradrenolutine, peak fluorescence appearing at 520–525 mμ for adrenolutine and 510–515 mμ for noradrenolutine with the particular spectrophotofluorimeters employed in this investigation. Furthermore, the relative fluorescence of noradrenolutine (compared to adrenolutine) was greatest for excitations in the 360–375 mμ region, and much less for excitations of 405 or 436 mμ (filter fluorimeter), or excitations near 340 mμ or in the 390–410 mμ region (spectrophotofluorimeter).

The absorption spectra of alkaline solutions of the lutines have also been presented.

Acknowledgement—The authors are indebted to Professor CHARLES R. DAWSON of the Department of Chemistry, Columbia University, for the loan of the special quartz cell employed for *in vacuo* optical density measurements.

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THE SIMULTANEOUS FLUORIMETRIC DETERMINATION OF ADRENALINE AND NORADRENALINE IN PLASMA—II*

PERIPHERAL VENOUS PLASMA CONCENTRATIONS IN NORMAL SUBJECTS AND IN PATIENTS WITH PHEOCHROMOCYTOMA

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(*Received* 23 *June* 1957)

INTRODUCTION

THE determination of adrenaline and noradrenaline in blood has been the subject of numerous investigations (PORAT, 1946; EULER and SCHMITERLÖW, 1947; GADDUM and LEMBECK, 1949; CRAWFORD and OUTSCHOORN, 1951; VOGT, 1952). Two methods which have received considerable attention are those reported by LUND (1949, 1950), and by WEIL-MALHERBE and BONE (1952,1953). In both of these methods, adrenaline and noradrenaline were extracted from serum or plasma by adsorption on to aluminium oxide, followed by elution with acid. Fluorescent derivatives were then formed and their concentrations fluorimetrically estimated. Adequate recoveries were reported with both methods. The essential difference between these two methods was the type of fluorescent derivative formed.

LUND converted adrenaline and noradrenaline into their corresponding 5:6-dihydroxyindoxyls (adrenolutine and noradrenolutine), by oxidation with manganese dioxide followed by tautomerization with alkali. Adrenaline and noradrenaline were differentiated by their oxidation characteristics at pH 6·5 and pH 3·0. Venous serum concentrations from normal human subjects were estimated as less than 1 μg adrenaline plus noradrenaline per litre (LUND, 1952).

WEIL-MALHERBE and BONE, on the other hand, formed fluorescent derivatives from adrenaline and noradrenaline by coupling them with ethylene diamine, a technique first reported for adrenaline by NATELSON, LUGOVOY and PINCUS (1949). Differences in the fluorescence emission spectra between the adrenaline and noradrenaline derivatives, permitted simultaneous determinations. Mean normal human plasma (venous) concentrations of 8·7 μg noradrenaline and 2·1 μg adrenaline per litre were reported (WEIL-MALHERBE and BONE, 1953).† The mean noradrenaline concentration was lowered roughly 13 per cent when further data had been accumulated (WEIL-MALHERBE and BONE, 1954). Mean values reported by others using modifications of the WEIL-MALHERBE and BONE procedure were: 2·1 $\pm$ 1·3 μg noradrenaline

* This work was supported by a grant from the U.S. Public Health Service, National Institutes of Health (Research Grant H-1045 (C5)).

† The blood values reported by WEIL-MALHERBE and BONE have been recalculated by us in order to express them in terms of μg per litre plasma. The method of calculation was that of WEIL-MALHERBE and BONE (1953); an average hematocrit value of 40 per cent was assumed.

and 0·4 ± 0·2 μg adrenaline per litre plasma (ARONOW, HOWARD and WOLFF, 1956); and 3·9 ± 1·7 μg noradrenaline and 0·14 ± 0·21 μg adrenaline per litre plasma (MANGER *et al.*, 1954).

The use of the lutines, rather than the ethylene diamine coupled derivatives was preferred by us for plasma analyses, since we were suspicious of the specificity of the latter technique. Our inability to duplicate LUND's (1950) preferential oxidation technique satisfactorily, led to the development of an alternate method for simultaneous adrenaline and noradrenaline determinations (COHEN and GOLDENBERG, 1957). In this present paper, the results obtained on application of this alternate procedure (multiple filter technique) to plasma are presented.

EXPERIMENTAL

A detailed description of the multiple filter technique for simultaneous fluorimetric adrenaline and noradrenaline analyses has been presented previously (COHEN and GOLDENBERG, 1957). Filter sets A and B employed for plasma analyses are identical to those utilized for demonstration purposes in the preceding paper.

Reagents. Isuprel hydrochloride (N-*iso*propyl noradrenaline hydrochloride) and 3-hydroxytyramine (dopamine) were obtained from the Sterling-Winthrop Research Institute, Rensselaer, New York. DL-β-(3 : 4-dihydroxyphenyl) alanine was obtained from Eastman Organic Chemicals, Rochester, New York, and 3:4-dihydroxyphenylacetic acid from Astra Ltd., Sodertalje, Sweden.*

Sterile liquaemin sodium (heparin), 1000 units per ml, was obtained from Organon Inc., Orange, New Jersey.

Aluminium oxide (alumina), non-alkaline, activity Grade 1 (M. Woelm-Eschwege, Germany) obtained from Alupharm Chemicals, New Orleans, La., was shaken in 200 to 300 g portions with one litre batches of 0·2 N-hydrochloric acid until the supernatant fluid was no longer turbid. The alumina was then washed several times with distilled water, 0·2 M-sodium acetate, again with distilled water, and finally air dried. Recoveries of adrenaline and noradrenaline were thus improved.

Reagent grade disodium phosphate was utilized without further purification. Other reagents were identical with those previously described.

Methods. Routinely, a 30 ml sample of peripheral venous blood (antecubital vein) was drawn into a syringe wetted with heparin solution. The syringe needle was removed and the sample slowly transferred to a cold 40 ml ground glass stoppered centrifuge tube, and therein stored in an ice-water bath. No more than six samples were handled at one time.

Cold blood specimens were centrifuged (700 G) at room temperature for 15 min. A measured volume of each supernatant plasma (generally 15 ml) was incubated with 0·3–0·4 g alumina for 3 min in 15 ml ground glass stoppered centrifuge tubes, with repeated inversion for mixing. An alumina sample (without added plasma) was taken through the purification procedure with each batch of plasma samples (see Discussion section, under Application of Method).

The samples were centrifuged about 1 min, and the supernatant plasma removed by means of a pipette attached to a water pump. The alumina samples were washed rapidly three times by the addition of 7 to 12 ml ice-cold distilled water with gentle agitation until all the alumina was dispersed, followed by removal of the supernatant liquid as before. Elution of adrenaline and noradrenaline was accomplished by the addition of a total of 6·0 ml 0·2 N-acetic acid (in two 3·0 ml portions), gentle dispersion and agitation of the alumina for 2 min, followed by centrifugation. It should be noted that during the preceding purification, alumina samples were allowed to settle for several seconds after agitation, and then those particles which had collected on the dry sides of the tube above the liquid level were rinsed down by swirling the supernatant fluid.

The eluates were transferred with elongated medicine droppers to calibrated 15 ml ground glass stoppered centrifuge tubes, and the pH of each specimen adjusted to 6·5–7·5 by the addition of 0·5 ml of 0·8 M-disodium phosphate, followed by 0·9 ml of 1 N-sodium hydroxide. The preliminary addition of disodium phosphate buffered the samples and prevented excessive local alkalinization upon the subsequent addition of sodium hydroxide. The sodium hydroxide was blown from a 1·0 ml pipette

* Kindly donated by Dr. R. DAHLBOM, of Astra, Ltd.

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(calibrated in 0·1 ml units) into the centre of each sample and the tube quickly capped and shaken to obtain rapid distribution of the alkali. In this manner, negligible premature lutine formation occurred in the strongly alkaline regions where autoxidation of adrenaline and noradrenaline could take place. Sample volumes were then adjusted to 8·0 ml with distilled water.

Samples were oxidized by the addition of 10–20 mg manganese dioxide followed by repeated inversion of the tubes for 1 min. After centrifugation for 1 to 2 min, each oxidized alumina extract was split into two equal portions: 0·5 ml of a 5:1 mixture of 5 N-sodium hydroxide and freshly prepared 10 per cent (w/v) L-ascorbic acid was added to one portion, and 0·5 ml of 5 N-sodium hydroxide to the other. The absence of ascorbic acid in the latter portion resulted in the decomposition of the lutines to non-fluorescent products; this sample provided an estimate of the contaminating fluorescence and was used as blank. The alkaline samples were centrifuged in order to spin down small amounts of alumina and/or manganese dioxide which were present. This sediment could be minimized by adequate centrifugation after the oxidation step.

The fluorimeter was standardized at 100 galvanometer deflection units (diaphragm no. 4 and filter sets *A* and *B*) with an 0·05 μg adrenaline standard. A standard of 0·05 μg noradrenaline and a reagent blank were also prepared for each set of plasma determinations. When plasma specimens contained more than 0·05 μg but less than 0·20 μg adrenaline and/or noradrenaline, the fluorimetric readings could be estimated by shifting to smaller diaphragms (nos. 5 or 6) and subsequently calibrating these diaphragms with the adrenaline standard. For samples containing more than 0·20 μg adrenaline and/or noradrenaline, dilution in an alkaline ascorbic acid solution facilitated analyses. Decomposed blanks were read after 25 min or longer had elapsed.

Between samples, the fluorimeter cell was rinsed with distilled water (generally three times) till a constant fluorimetric reading was obtained (6·5–8·0 deflection units with filter set *A*, and 1·5–2·0 deflection units with filter set *B*). The empty fluorimeter cell registered 1·0–1·5 deflection units with filter set *A* and 0·25–0·50 deflection units with filter set *B*.

Glassware. All glassware was scrupulously cleaned with soap and water, rinsed in dilute acid to remove traces of soap, and then rinsed with distilled water. The 15 ml centrifuge tubes and ground glass caps employed for pH adjustment of plasma extracts and subsequent oxidation with manganese dioxide, were soaked in concentrated hydrochloric acid to remove traces of manganese dioxide embedded in scratch marks and ground glass surfaces. Premature oxidation of adrenaline and noradrenaline by such traces was thus avoided.

As a special precaution the 15 ml centrifuge tubes and ground glass caps employed for incubation of plasma and alumina were kept separated from those tubes utilized for the oxidation step. Syringes, needles, and 40 ml centrifuge tubes and caps were also guarded against possible contamination with manganese dioxide.

RESULTS

The concentrations of adrenaline and noradrenaline in peripheral venous plasma obtained from fifty-nine non-hypertensive volunteers (medical students and hospital employees) were fluorimetrically determined. Forty-seven samples were obtained from males and eighteen from females, for a total of sixty-five samples; included are several males on whom repeat tests were run on different occasions. Of these sixty-five samples, fifty-one were obtained from individuals between the ages of 20 and 30 years, and fourteen from individuals between the ages of 32 and 51 years.

Plasma concentrations ranged from 0·09 to 0·50 μg noradrenaline per litre and from —0·05 to 0·19 μg adrenaline per litre. Small negative values for adrenaline were within the precision of the method. The following mean values and average deviations were calculated; $0{\cdot}30 \pm 0{\cdot}07$ μg noradrenaline and $0{\cdot}06 \pm 0{\cdot}05$ μg adrenaline per litre plasma.

Recoveries of 0·03–0·05 μg adrenaline and/or noradrenaline added to sample portions of various plasma specimens varied in the range of from 70 to 90 per cent. Duplicate recoveries, or duplicate plasma specimens without added material, agreed to within 2 galvanometer deflection units for readings of 50–60 galvanometer

deflection units, and to within 3 deflection units for higher readings. Duplicate samples of standards agreed to within 1 to 2 deflection units over the complete range, as reported in the preceding paper (COHEN and GOLDENBERG, 1957). Recoveries

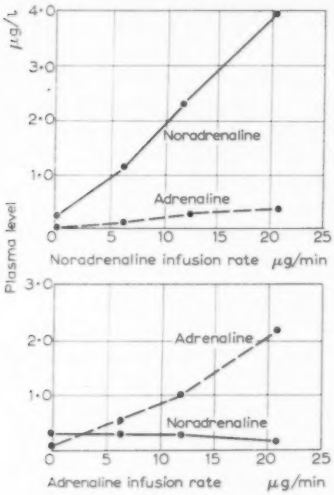

FIG. 1.—Observed plasma levels for several infusion rates. At each infusion rate, samples were drawn after 10 min had elapsed. Plasma levels are uncorrected for recoveries. Upper curves: A. F., male, 21 years old, 185 lb. Lower curves: R. D., male, 23 years old, 135 lb.

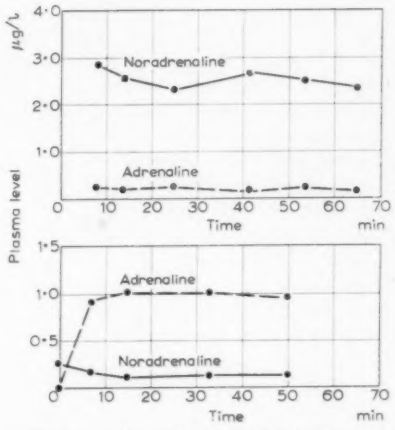

FIG. 2.—Observed plasma levels for constant rate of infusions. Plasma levels are uncorrected for recoveries. Upper curves: A. K., male, 21 years old, 175 lb, 20·8 μg noradrenaline infused per minute. Lower curves: E. B. male, 23 years old, 165lb, 12·4 μg adrenaline infused per minute.

TABLE 1.—PLASMA LEVELS IN PHEOCHROMOCYTOMA

Patient	Type of hypertension	Blood sample	Plasma concentration*	
			Noradrenaline (μg/l.)	Adrenaline (μg/l.)
R.S.	persistent	at rest	5·9	2·6
J.B.	persistent	at rest	30·5	0·2
M.B.	persistent	1, at rest	2·8	0·3
		2, during anesthesia	48·0	2·9
M.L.	paroxysmal	1, at rest	0·57	0·25
		2, during paroxysm	20·9	0·1
D.S.	paroxysmal	1, at rest	0·82	0·39
		2, following histamine	42·0	42·2
		3, during surgery	96·8	25·4

* Uncorrected for recoveries of 70–90 per cent.

run with whole blood indicated that not more than a 5–10 per cent loss of added material was incurred during incubation of the samples in ice water for 30–60 min, followed by centrifugation of the red blood cells.

Intravenous infusions of adrenaline and noradrenaline were performed, the details of which are reported elsewhere (COHEN, HOLLAND and GOLDENBERG, in preparation), and from which the sample data of Figs. 1 and 2 have been abstracted. Blood samples were drawn from the right antecubital vein of normal volunteers, during infusions in the left antecubital vein.

TABLE 2.—SAMPLE DATA AND CALCULATIONS

Sample	Galvanometer deflections			
	Filter set *A*		Filter set *B*	
	Reading	Corrected for blank	Reading	Corrected for blank
0·05 μg Adrenaline	100·00	86·75	100·00	93·00
0·05 μg Noradrenaline	91·00	77·75	24·00	17·00
Reagents	13·25		7·00	
Normal	26·00	9·00	15·50	6·50
Decomposed blank	17·00		9·00	
Pheochromocytoma	76·25	52·00	33·00	20·00
Decomposed blank	24·25		13·00	
Alumina	15·00	3·50	10·00	5·00
Decomposed blank	11·50		5·00	

$$\text{Noradrenaline } (\mu g \text{ per sample}) = y = \frac{(AE_b/E_a) - B}{(N_aE_b/E_a) - N_b}$$

$$\text{Adrenaline } (\mu g \text{ per sample}) = x = \frac{B - yN_b}{E_b}$$

E_a, E_b, N_a, and N_b are the fluorescence per μg on filter sets *A* and *B*, for the adrenaline and noradrenaline standards, respectively. A and B are the fluorimetric readings (ascorbic acid stabilized) for each sample on filter sets *A* and *B*, respectively, corrected for the fluorescence of the decomposed blanks (see table), and for the fluorescence of the alumina reagent blank (see below).

$E_a = 86{\cdot}75/0{\cdot}05\ \mu g$ $\quad N_a = 77{\cdot}75/0{\cdot}05\ \mu g$
$E_b = 93{\cdot}00/0{\cdot}05\ \mu g$ $\quad N_b = 17{\cdot}00/0{\cdot}05\ \mu g$

(1) Normal plasma specimen (15·0 ml)

$A = 9{\cdot}00 - 3{\cdot}50 = 5{\cdot}50$ $\quad$ * Noradrenaline = 0·22 μg/l.
$B = 6{\cdot}50 - 5{\cdot}00 = 1{\cdot}50$ $\quad$ * Adrenaline = 0·01 μg/l.

(2) Pheochromocytoma plasma specimen (10·0 ml)

$A = 52{\cdot}00 - 3{\cdot}50 = 48{\cdot}50$ $\quad$ * Noradrenaline = 2·8 μg/l.
$B = 20{\cdot}00 - 5{\cdot}00 = 15{\cdot}00$ $\quad$ * Adrenaline = 0·3 μg/l.

*Plasma concentrations uncorrected for recoveries.

Straight line plots of plasma concentrations versus infusion rates were obtained (Fig. 1). It should be noted that the concentration of adrenaline remained essentially constant during intravenous infusions of noradrenaline, and vice versa. Prolonged constant rate infusions (Fig. 2) indicated no trend towards increased concentration with time, i.e. essentially constant levels were observed.

Plasma concentrations of adrenaline and noradrenaline were also studied in fourteen patients with pheochromocytoma (chromaffin tumors which produce and secrete noradrenaline and sometimes adrenaline also). Representative data are presented in Table 1. Included are samples taken during spontaneous and histamine-induced paroxysms, anesthesia, and surgery. For each of the individuals the elevated plasma levels were correlated with similar increases in urinary excretion and with the noradrenaline and adrenaline content of the excised tumours, by the techniques of GOLDENBERG *et al.* (1954).

Sample fluorimetric data are presented in Table 2 for one pheochromocytoma plasma specimen and one normal plasma specimen, to illustrate the magnitude of fluorescence readings and sample calculations.

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DISCUSSION

Instrumentation

In the preceding paper (COHEN and GOLDENBERG, 1957), the fluorescence characteristics of adrenolutine and noradrenolutine were studied in detail. Methods for the simultaneous determination of adrenaline and noradrenaline were presented, based upon differences between the excitation and fluorescence emission spectra of their lutines.

For the analysis of the small amounts of adrenaline and noradrenaline found in plasma, the Farrand filter fluorimeter Model A was found to be more suitable than a spectrophotofluorimeter with regard to sensitivity and stability. The choice of filter combinations was dependent upon the high sensitivities required for plasma analyses and the fluorescence characteristics of contaminants. Filter combinations which resulted in relatively strong readings from the reagents, either as a result of fluorescence of the reagents or filters, or the transmission of activating radiation due to light scattering, could not be utilized. Eliminated for these reasons were most of the secondary filters which isolated a proportionately greater section of the noradrenolutine fluorescence than that of adrenolutine (COHEN and GOLDENBERG, 1957).

The intensities of fluorescence, corrected for blanks, elicited from plasma eluates by separate excitations of 365, 405 and 436 mμ were compared. The amount of adrenaline and noradrenaline indicated by 365 mμ excitations were generally greater than those indicated by either 405 or 436 mμ excitations. Since it could be demonstrated that the discrepancy was not due to the inhibition of fluorescence for 405 and 436 mμ excitations, it followed that the excessive fluorescence (365 mμ excitations) was due to contaminants. For this reason, 365 mμ excitations could not be employed. On the other hand, the fluorescence obtained with 405 and 436 mμ excitations agreed well.

Comparison of lutine and ethylene diamine procedures

The normal plasma (venous) levels of adrenaline and noradrenaline which we

have recorded (see Results) are significantly lower than those previously reported by others with the ethylene diamine procedure (see Introduction). Our lower values cannot be attributed to losses since adequate recoveries of added material have been demonstrated. It is our opinion that the discrepancy between the two methods is due to a lack of specificity in the ethylene diamine procedure.

Results similar to ours have been recently reported (VALK and PRICE, 1956); the fluorimetric method employed therein appears closely related to ours. The specificity of the ethylene diamine procedure has been questioned by others (HOLZBAUER and VOGT, 1954).

In both the lutine and ethylene diamine procedures, adrenaline and noradrenaline are isolated by adsorption on to alumina followed by elution with acetic acid. Unfortunately, this purification is not specific for adrenaline and noradrenaline, but is a general method applicable to the isolation of catechols and most probably di-enols also.

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The reaction of ethylene diamine with an alumina eluate of plasma is again a non-specific procedure applicable to catechols in general, subsequent *iso*butanol extraction isolating basic and neutral compounds. It is evident therefore, that any adrenaline and noradrenaline specificity of the ethylene diamine procedure is dependent upon the absence in plasma of significant quantities of other catechols which could form extractable ethylene diamine derivatives.

Of particular interest in this connexion is the compound, β-(3:4-dihydroxyphenyl) ethylamine, i.e. 3-hydroxytyramine or dopamine, a normal constituent of urine, of which 100–200 μg are excreted per 24 hr (EULER, HAMBERG and HELLNER, 1951); these amounts are greater than those of noradrenaline, of which 14–41 μg are excreted per 24 hr (GOLDENBERG *et al.*, 1954). The presence of dopamine in plasma in a similar concentration ratio relative to noradrenaline, would result in gross overestimations with the ethylene diamine procedure.

In comparison, the lutine procedure yields fluorescent derivatives from alumina isolated compounds in a much more specific manner. The reaction appears dependent upon the formation of fluorescent indole nuclei from catechol amines; the fluorescent derivatives formed from adrenaline and noradrenaline are 3:5:6-trihydroxyindoles. Furthermore, when ascorbic acid stabilization is omitted in aliquot portions of the extracts, the decomposition of adrenolutine and noradrenolutine to non-fluorescent products provides an estimate of contaminating fluorescence. No comparable blanks are available in the ethylene diamine procedure.

Catechol compounds possessing ethylamine side-chains (dopamine and β-(3:4-dihydroxyphenyl)alanine, i.e. 3:4-dopa), or compounds possessing ethanolamine side-chains (N-*iso*propyl noradrenaline), represent two types of potentially interfering substances (Table 3). However, neither significant quantities of N-*iso*propyl noradrenaline nor any other catechols with ethanolamine side-chains have been demonstrated in man; therefore, this type of compound probably does not represent a major source of error. With regard to 3:4-dopa, its fluorescence per μg is less than one-twentieth that exhibited by adrenaline or noradrenaline with the particular filters used (Table 3). Concentrations of 3:4-dopa in plasma equal to that of noradrenaline could account for only 4 per cent of the noradrenaline levels herein reported; higher concentrations of 3:4 dopa, if present, would interfere to greater extents. Dopamine, in comparison, fluoresces even more weakly (Table 3). If the maximum concentration of dopamine

in plasma is assumed to be proportional to its average urinary concentration relative to noradrenaline, a ratio of, roughly 150 μg : 28 μg, or 5·5 : 1, then dopamine could account for no more than 7 per cent of the noradrenaline levels.

Experimental indication of the specificity of the lutine procedure is afforded by the results obtained during intravenous infusions. First, the ability to differentiate between adrenaline and noradrenaline is indicated by the finding that infused adrenaline appeared fluorometrically, not as a mixture, but solely as adrenaline. The same

TABLE 3.—FLUORESCENCE OF SEVERAL CATECHOL COMPOUNDS
Lutine procedure, i.e. oxidation with manganese dioxide followed by addition of 5 N-sodium hydroxide-ascorbic acid mixture

Compound	Sample μg	Galvanometer deflections	
		Filter set *A*	Filter set *B*
Adrenaline	0·1	100·0	100·0
Noradrenaline	0·1	92·0	24·0
N-*iso*propyl noradrenaline	0·1	77·0	75·5
3:4 dopa	2·0	72·0	5·0
Dopamine*	4·0	50·0	10·0
3:4-dihydroxyphenylacetic acid	5·0	9·5	3·5
Reagents	—	7·0	4·0

* Dopamine fluorescence was variable. A relatively strongly fluorescent sample is presented in the table for discussion regarding possible interference with plasma analyses of noradrenaline and adrenaline.

held true for noradrenaline. Secondly, the linear relationship between plasma levels and infusion rates (Fig. 1), as well as the constant levels observed during infusions at constant rate (Fig. 2), argues against the accumulation of fluorimetrically observable metabolites of adrenaline and noradrenaline. The accumulation of such metabolites would have resulted in plasma levels which increased with time in Fig. 2, and given an exponentially rising curve in Fig. 1.

In consideration of the probable role of amine oxidase in the inactivation of adrenaline and noradrenaline (BLASCHKO, 1952), the anticipated metabolites would not be expected to produce fluorescent contaminants in the lutine procedure. The absence of amino functions precludes the formation of fluorescent indoles from such compounds as 3:4-dihydroxyphenyl-glycolaldehyde (amine oxidase action), and 3:4-dihydroxymandelic acid (subsequent aldehyde oxidase action). In Table 3 the lack of fluorescence is demonstrated for 3:4-dihydroxyphenylacetic acid, a compound which may be derived from dopamine by the same pathway.

In contrast with the lutine procedure, compounds such as 3:4-dihydroxyphenyl-glycolaldehyde contain the requisite catechol structure for reaction with ethylene diamine, and the products should be extractable into *iso*butanol. Although ionized carboxylic groups of acids prevent extraction of the parent compounds from alkaline media, the extraction of fluorescent material derived from 3:4-dihydroxyphenylacetic acid has been recently reported (VALK and PRICE, 1956). This phenomenon may have resulted from loss of the acid function through decarboxylation.

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In conclusion, the elevated estimates of plasma levels obtained with the ethylene diamine procedure may be attributed to the probable presence in plasma of any one, or a combination of a variety of interfering compounds. The lutine procedure, on the other hand, is less subject to contamination from these sources.

Application of method. It should be recalled that the multiple filter procedure herein employed differs from LUND's (1950) technique of varying the ratio of fluorescence obtained from adrenaline and noradrenaline by oxidations in neutral and acidic media. This latter procedure was less convenient and more time-consuming since two pH adjustments as well as four aliquots (ascorbic acid stabilized sample and decomposed sodium hydroxide blank, at each pH) were required from each plasma specimen, as compared to a single pH adjustment and only two aliquots for the multiple filter procedure. The increased sensitivity obtained by working with a smaller number of aliquots was of major importance.

Two examples of plasma analyses have been presented in Table 2. A special reagent blank consisting of an alumina sample taken through the entire procedure was required for each group of analyses, to correct for the fluorescence of ascorbic acid itself, and also that devoloped by reaction of ascorbic acid with the reagents. This type of blank was not necessary at lower sensitivity settings, such as that employed with urinary extracts (GOLDENBERG *et al.*, 1954) where the effect of ascorbic acid was not as noticeable. Fluorescence readings for each plasma sample were corrected for those of its corresponding sodium hydroxide blank and for the alumina reagent blank.

It should be noted that the small amounts of fluorescence above blanks detected in 'normal' plasma specimens had associated with them a low degree of precision and reliability. Furthermore, the possibility could not be disregarded that the 'normal' plasma levels were elevated to varying degrees by fear and apprehension on the parts of the subjects. Therefore, although true 'normal' levels may be even lower, extending below the sensitivity of the method in its present stage of development, certainly in view of the recoveries of added adrenaline and noradrenaline, they can be no higher than herein reported.

The relatively small readings above blanks obtained from normal subjects preclude the application of the described procedure to situations in which even lower plasma levels may be anticipated, e.g. the possible effect of some tranquilizing agents. However, we have applied the procedure with success in situations in which increased plasma levels of adrenaline and noradrenaline were anticipated.

In pheochromocytoma, we have found significantly elevated plasma levels (Table 1) to be of diagnostic value. In the paroxysmal hypertensive variety, the levels obtained during spontaneous or histamine induced paroxysms were markedly higher than those exhibited by the persistent hypertensive variety. The inherent danger of the histamine test is demonstrated in the sudden and marked elevation of plasma levels. High levels were also observed during anesthesia and surgery.

SUMMARY

(1) The multiple filter technique for the simultaneous fluorimetric analysis of adrenolutine and noradrenolutine has been applied to the determination of plasma levels of adrenaline and noradrenaline. Mean normal values (uncorrected for 70–90 per cent recoveries) of 0·30 $\pm$ 0·07 (A.D.) μg noradrenaline and 0·06 $\pm$ 0·05 (A.D.) μg adrenaline per litre plasma from peripheral venous blood were obtained.

(2) These values were significantly lower than those previously reported by others working with the ethylene diamine method. Since adequate recoveries of adrenaline and noradrenaline from plasma and whole blood were obtained, our smaller values could not be attributed to losses. The ethylene diamine procedure therefore appears to be substantially in error due to lack of specificity for adrenaline and noradrenaline. This problem is discussed in detail.

(3) Elevated plasma levels of noradrenaline and adrenaline were detected in patients with pheochromocytoma. Representative data have been presented for patients at rest, and also during anesthesia, surgery, and both spontaneous and histamine-induced paroxysms.

Acknowledgement—The technical assistance of Mr. OSCAR BING and Miss JOANNE YUNG is gratefully acknowledged.

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Journal of Neurochemistry, 1958, Vol. 2, pp. 81–101. Pergamon Press Ltd., London

OXYGEN CONSUMPTION OF EXCISED SYMPATHETIC GANGLIA AT REST AND IN ACTIVITY*

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(*Received* 9 *May* 1957)

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SYMPATHETIC ganglia of mammals have recently been found suitable for a variety of metabolic studies. By the use of these ganglia metabolism can be measured in a preparation which contains nerve cell bodies and synapses, and which survives for long periods after excision when kept in appropriate solutions at or below normal body temperature. Thus well-controlled measurements can be made under reproducible conditions, simultaneously with electrical tests of axonal conduction and synaptic transmission, and with nerve cells at rest or in repetitive action as desired by the experimenter.

The superior cervical ganglion of the rat has been chosen for a number of studies in this laboratory, some of which have already been published (LARRABEE and BRONK, 1952; EDWARDS and LARRABEE, 1955). It is the purpose of this paper, first to describe an improved method for determining the rate of oxygen uptake of these preparations, secondly to report measurements of oxygen uptake at rest and during various frequencies of stimulation, and finally to compare certain of the results with those previously reported by others for frog nerve. These data will subsequently be compared with observations on ganglionic glucose uptake and lactate production (HOROWICZ and LARRABEE, 1957).

METHODS

The respirometer to be described permits measurement of the rate of oxygen uptake by small amounts of tissue under sterile conditions. Sterile conditions were needed, because otherwise a respirometer which contained no ganglion was found to consume considerable oxygen, sometimes as much as the ganglion preparation itself, which typically had a wet weight of only about one milligram.

The respirometer was an adaptation of the continuous flow instrument developed for use on nerve trunks by CARLSON, BRINK and BRONK (1950). In this method a suitably polarized platinum electrode (DAVIES and BRINK, 1942) is used to measure the amount of oxygen remaining in a solution which has flowed at a known rate through a small chamber containing the tissue. The absolute rate of oxygen uptake can be calculated from the rate of flow of solution and the concentrations of oxygen in the fluids entering and leaving the respirometer. The details of this calculation will be explained at the end of the methods section of this paper, after first describing the preparation, the apparatus, and various experimental procedures.

* This investigation was supported in part by a research grant (No. B–702) from the National Institute of Neurological Diseases and Blindness of the National Institutes of Health, United States Public Health Service. Preliminary reports of this work and preliminary comparisons with glucose and lactate measurements have been published by DOLIVO *et al.* (1956), LARRABEE and HOROWICZ (1956), and LARRABEE *et al.* (1957).

Preparation. Superior cervical ganglia, along with suitable lengths of preganglionic and postganglionic nerves, were excised aseptically from adult hooded rats of both sexes (Lashley strain), with body weights usually between 180 and 220 g. The excision was done under urethane anaesthesia because of previous evidence that this agent has less effect than others on sympathetic ganglia when used in concentrations required for surgical anaesthesia (LARRABEE and HOLADAY, 1952; DEMPSHER, LARRABEE, BANG and BODIAN, 1955). The ganglia were placed immediately in sterile bathing solution at room temperature and transferred into the respirometer with precautions against contamination. At the end of the experiment the preparation was dried to constant weight by placing it in an oven at 90 to 95°C for at least ½ hr. The dry weight was usually about 0·2 mg (Table 1). Wet weights were

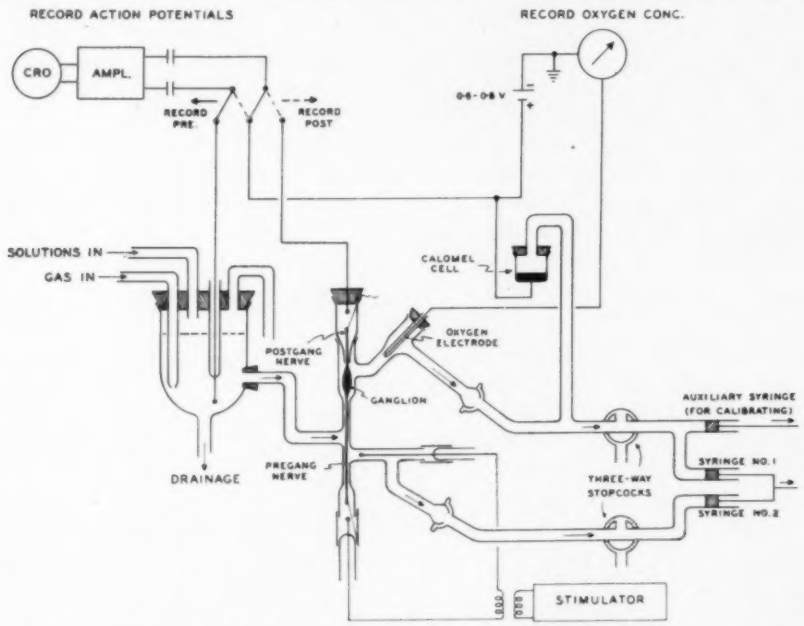

FIG. 2.—Diagram of the respirometer, showing electrical connexions, etc.

also measured on some preparations after blotting on filter paper. Ratios of wet weights to dry weights were found to average 4·7 ± 0·1 (mean ± S.E.M.) on 13 preparations by one observer and 4·2 ± 0·4 on 49 preparations by another. About 5 mm of preganglionic nerve was included in the measurements of oxygen uptake and dry weight. This quantity of nerve, weighed separately in four preparations, contributed 15, 19, 23, and 24 per cent of the total dry weight. Postganglionic nerves were cut off and discarded before weighing, since the position of these nerves in the respirometer made it unlikely that they significantly affected the oxygen measurements; usually they were not more than 2 or 3 mm in length.

Respirometer. The tissue chamber of the respirometer for rat ganglia is shown in Figs. 1 and 2. The platinum electrode for measuring oxygen concentration was inserted through a stopper, so that its tip was in the solution leaving the ganglion. Electrodes were provided for stimulating the preganglionic nerve and recording from the preganglionic and postganglionic nerves. It should be noted that solution which had gone past the stimulating electrodes and the nearby portion of the nerve was carried away by syringe no. 2 and therefore did not affect the oxygen measurements, since these were made in the outflow to syringe no. 1. Therefore changes in oxygen uptake caused by stimulation were effects of conducted impulses and were not direct effects of the stimulating current.

In addition to the glass parts shown in Fig. 1, there was an assembly of four two-way stopcocks and four 125 ml Erlenmeyer flasks. This assembly terminated in a delivery tube which projected through a stopper into the top of the reservoir. By manipulating the stopcocks with the aid of extension handles, and by raising the pressure of the gas over the fluids contained in the flasks, solutions

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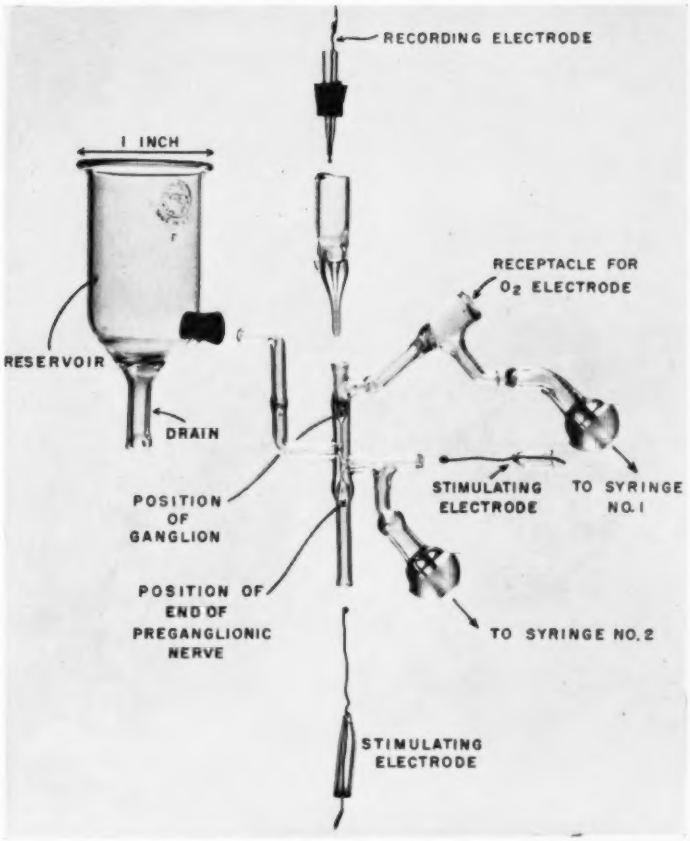

FIG. 1.—Tissue chamber of the respirometer for measuring rate of oxygen consumption by superior cervical ganglia of rats.

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could be transferred into the reservoir without opening the temperature box in which the apparatus was kept during an experiment. The reservoir could also be drained from outside the box.

Syringe drive. The motor-driven dual syringe for controlling rate of flow through the respirometer was designed and constructed in this laboratory by Mr. J. P. HERVEY, Dr. C. M. CONNELLY and Mr. W. BITTERLICH. A synchronous motor rotated a precision-ground screw, which in turn moved the plungers of the syringes. The syringes consisted of precision-ground glass tubes with close-fitting stainless steel plungers (purchased from Fischer and Porter Co. of Hatboro, Pa.). Moving the plungers at the rate of 1·52 cm/hr caused solution to flow past the ganglion at a rate of 0·30 to 0·75 ml/hr with the various sizes of syringes that were used. The total travel of over 23 cm permitted an experiment to run for more than 15 hr without resetting the syringes.

Oxygen electrode. The electrode used for measuring oxygen concentration consisted of a piece of platinum wire, 1 or 2 thousandths of an inch in diameter, sealed into an insulating sleeve of glass. The tip of the electrode was ground back flush with the end of the glass. The end of the glass along with the exposed surface of the platinum, was coated with a layer of collodion, which was of such a thickness that the sensitivity of the electrode was reduced several fold. A coating was necessary in order to stabilize the electrode current in a bicarbonate-buffered solution (LARRABEE *et al.*, 1952). It also made the electrode current relatively insensitive to changes in rate of flow of the bathing fluid, and thus simplified the record when the flow was accelerated for purposes of calibration, as described below.

The electrode was maintained 0·6–0·8 v negative with respect to the calomel cell through which the circuit was completed. Tests showed that under these conditions the electrode current was directly proportional to oxygen concentration (DAVIES and BRINK, 1942; CARLSON *et al.*, 1950).

Position of oxygen electrode. The concentration of oxygen was obviously not the same at all points in the fluid leaving the tissue chamber, since more oxygen must have been removed from fractions of the volume which passed near the tissue than from fractions which passed near the glass walls. These non-uniformities in oxygen distribution were progressively reduced by diffusion of oxygen as the fluid slowly moved along the tube leading to the oxygen electrode. It was essential to allow enough distance between the tissue and the electrode, so that the concentration of oxygen could be assumed uniform throughout the cross-section of fluid at the electrode tip when calculating oxygen uptake. CARLSON *et al.* (1950) have derived an equation for calculating an electrode position at which the oxygen concentration at all points in the cross-section will differ less than 2 per cent from the uniform concentration achieved at infinite distance. Application of their criterion to our apparatus indicated that the distance from tissue to the electrode never needed to be more than about 1·6 mm. Since the actual distance exceeded 10 mm, uniform concentration throughout the cross-section could safely be assumed. This distance might have been shortened and some of the time lags in the method might thereby have been reduced, but the existing time lag of several minutes was convenient for certain calibration procedures described below.

Amplifiers and stimulator. The nerve action potentials were recorded with a capacity-coupled amplifier and cathode-ray oscilloscope. The amplifier was connected to the preparation through input capacitors, because of differences between the steady potentials of the platinum and calomel electrodes through which the responses were recorded.

Stimuli were brief condenser-discharge shocks delivered to the preganglionic nerve through an isolating transformer, or rectangular pulses of 0·1–2 msec duration delivered through a radio-frequency isolation unit. Stimuli were adjusted to a strength which produced a postganglionic action potential of maximal or near maximal height.

In the earlier experiments, the oxygen electrode current was passed through a resistor and the resulting voltage drop was amplified with a 'chopper' amplifier or with a conventional direct-coupled amplifier. Later this system was replaced with one in which the current from the electrode was led to the grid of the input tube of a direct-coupled amplifier, but was drawn off through a feedback resistor connected to the output of the amplifier (CHANCE, HUGHES, MACNICHOL, SAYRE and WILLIAMS, 1949). This arrangement had advantages in simplicity, very great stability, and constancy of voltage at the input terminal. Outputs of the various oxygen-electrode amplifiers were recorded on a General Electric recording microammeter of the printer-bar type.

Provisions were made for displacing the zero of the oxygen electrode current off of the recording paper, so that changes in electrode current could be recorded at increased amplification. Provision was also made for automatically disconnecting the oxygen electrode circuit from the amplifier input

at regular intervals, so that the baseline of the amplifier could be recorded. This was done without interrupting the electrode current, except momentarily during switching.

Temperature. Experiments were conducted in a thermostated box, at temperatures between 23 and 37°c. Because the oxygen electrode current was sensitive to changes in temperature, the smoothness of the records was improved by minimizing temperature excursions during the thermostat cycle. For this purpose only a small fraction of the total heat supply to the box was directly controlled by the thermostat, the rest of the heater being automatically adjusted by a motor-driven controller in such a way that the thermostat was on approximately 50 per cent of the time.

Solutions. The bathing solution contained the following materials: 136 mM-NaCl, 5·6 mM-KCl, 2·22 mM-$CaCl_2$, 1·2 mM-$MgCl_2$, 1·2 mM-NaH_2PO_4, 16·2 mM-$NaHCO_3$, 5·5 mM-glucose. The solution was equilibrated with a gas containing 5 per cent CO_2 and 95 per cent O_2 except where noted. This gas bubbled through the reservoir and through the reserve flasks throughout the experiment, giving a pH of 7·3–7·4 at 36°. Solutions were at first made with water which had passed through a demineralizer after preparation in a metal still, later with water prepared directly in a glass still of high quality (BALLANTINE, 1954), and finally with water prepared in a metal still and then redistilled in 'Pyrex' glass.

The solution was made sterile as follows: (a) A stock solution was prepared containing NaCl, KCl, $NaHCO_3$, and NaH_2PO_4 in 10 times the final concentrations. Ten ml volumes of this concentrated solution, together with 88 ml of distilled water, were placed in 125 ml Erlenmeyer flasks. Each flask was closed with a stopper carrying two glass tubes for bubbling gases through the solution, the inlet tube having a cotton plug to prevent contamination. After covering the tops, the flasks were sterilized in an autoclave. (b) $CaCl_2$ and $MgCl_2$ were prepared separately as 2M solutions, the concentrations being adjusted with the aid of a hydrometer. A mixture of appropriate dilution was then prepared and 2 ml volumes were sterilized by autoclave in small test tubes, each of which contained the correct amount of these two substances for addition to one of the flasks described above. (c) Glucose solution was prepared in a concentration of 100 mg/ml and sterilized, either in an autoclave or by passing through a fritted glass filter of 'ultra-fine' porosity. One ml volumes were placed in sterile test tubes. (d) All solutions were stored in the refrigerator after sterilizing.

On the day of the experiment, the solution in the required number of flasks was bubbled with 5 per cent CO_2, 95 per cent O_2 for at least 10 min. The $CaCl_2$, $MgCl_2$, and glucose were then added with sterile precautions. Further equilibration with the gases was resumed after the solutions were in the temperature box and was continued throughout the experiment.

Preparing the apparatus for an experiment. The respirometer and reservoir (Fig. 1) were filled with unbuffered Ringer's solution after stoppering the various outlets. The stopcock assembly, with flasks attached, was put in place, gas inlets and vents were closed with cotton plugs, and the whole apparatus sterilized in an autoclave.

Sterile solutions, as needed for the experiment, were then introduced into the flasks and into the reservoir, and the apparatus was placed in the temperature box. Connexions were made to the motor-driven syringes by means of the ball and socket joints shown in Fig. 1, and to the equilibrating gas. The oxygen electrode, which did not have to be sterilized because it was down stream from the region in which contamination could be harmful, was next inserted. An hour or more was then allowed for equilibration of gas in the solutions and for stabilization of the oxygen electrode. Finally the preparation was introduced aseptically with the aid of threads which had been strung through the respirometer before autoclaving.

Calculation of rate of oxygen consumption. The calculation was based on the following relationship: (rate of oxygen uptake) = (amount of oxygen entering the respirometer chamber per unit time) × (fraction of oxygen removed by tissue).

The amount of oxygen entering the respirometer was given by the following product: (rate of volume flow of solution to motor-driven syringe no. 1) × (the concentration of oxygen in the solution in the reservoir).

The concentration of oxygen (in moles/ml) was equal to: (q/3200) × (0·95) × (the absolute density of water in g/ml at the experimental temperature) × (the fraction of O_2 in the equilibrating gas.) The letter q represents the g of O_2 dissolved in 100 g of H_2O when the partial pressure of O_2 plus the aqueous vapour tension equals 760 mm, as tabulated in the *Handbook of Physics and Chemistry.*

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The factor 0·95 was introduced to allow for the reduction of O_2 solubility caused by salts in the solution. The magnitude of this factor was estimated for a solution containing 150 mM-NaCl from data in the International Critical Tables, using logarithmic interpolation as suggested by RANDALL and FAILEY (1927). These sources indicate that the factor is essentially independent of temperature.

The variable which had to be determined experimentally was the fraction of oxygen removed by the tissue. This will be represented by the letter F.

By definition: $$F = (C_1 - C_2)/C_1,$$

where C_1 and C_2 represent the concentrations of oxygen entering and leaving the respirometer respectively. According to Henry's law the amount of oxygen in solution at fixed temperature may be assumed proportional to the partial pressure of oxygen.

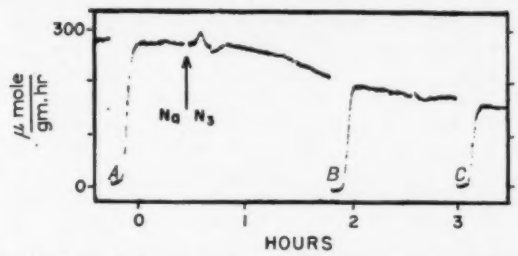

FIG. 3.—A typical record of oxygen uptake. Deflections at *A*, *B*, and *C* were caused by acceleration of flow rate for purposes of calibration, as described in the text. Concentration of sodium azide was 0·5 mM. 36°C.

Moreover it was shown by frequent tests that the deflection on the recording paper was directly proportional to the partial pressure of oxygen. In other words,

$$C \propto \text{partial pressure} \propto D$$

Therefore $$F = (D_1 - D_2)/D_1$$

where D_1 and D_2 are the deflections corresponding to the concentrations of oxygen entering and leaving the respirometer respectively.

Recording procedures are best understood by reference to a typical tracing (Fig. 3). In all of our records the oxygen scale is inverted, with zero oxygen concentration at the top, usually displaced considerably above the top of the recording paper. Accordingly an upward deflection signifies that less oxygen is reaching the electrode and therefore that the rate of consumption has increased. An ordinate corresponding to the oxygen concentration entering the respirometer (D_1) was determined occasionally during an experiment by greatly accelerating the flow of solution, so that only a negligible fraction of the oxygen was removed by the tissue (at *A*, *B*, and *C* in Fig. 3). This ordinate of course represented not only the concentration of oxygen in the reservoir but also zero rate of oxygen consumption. Values of this ordinate at other times were found by linear interpolation, usually by ruling straight line segments between successive recordings of D_1 on the original record. Displacement above this line was equal to $(D_1 - D_2)$ and was thus directly proportional to the rate of O_2 uptake (except when the record was disturbed during and immediately after recording D_1). This made it possible to obtain information about changes in rate of oxygen uptake by direct examination of the record. For more quantitative purposes D_1 and D_2 were read from the record and calculations were made by formulae indicated above. Changes

in sensitivity of the oxygen electrode, which sometimes occurred, were revealed by changes in D_1 and were thus automatically allowed for by the procedures described.

In the earliest experiments the acceleration of flow was obtained by letting solution run out through a stopcock on the tubing leading to syringe no. 1. In later experiments the accelerations were caused more reproducibly by an auxiliary motor-driven syringe connected to the same tubing (Fig. 2). This also made it possible to allow for a small error which was neglected in formulating the above equations: this error resulted from the fact that the rate of the accelerated flow was not actually infinite, so that the accompanying deflection (D_1) did not correspond exactly to the concentration in the reservoir. It can be shown that correction for this error is made by calculating F as before from the recorded D_1 and D_2 and then dividing this F by a factor:

$$\text{Correction factor} = 1 - (S/S_e)(1 - F)$$

where S = flow rate for syringe no. 1, and S_e = flow rate for the auxiliary syringe plus syringe no. 1. This correction increased the calculated rate of oxygen uptake by less than 2 per cent under the conditions of our experiments and might reasonably have been neglected. Nevertheless the correction was applied to all data in Table 1 obtained after the auxiliary syringe was added in September, 1955.

Controls on sterile conditions. The purpose of employing sterile procedures was to keep the rate of oxygen utilization by micro-organisms down to a negligible level. This was considered to be satisfactorily achieved whenever the rate of uptake by the empty respirometer and the solutions within it were found to be negligible after the tissue had been removed at the end of the experiment. This condition usually was satisfied even in experiments lasting 8 or more hours at 36°C.

When significant contamination of the bathing fluid developed during the course of the experiment, this usually could be detected at the times when the flow was accelerated for purposes of calibration. At *A, B, C* in Fig. 3, for example, rapid flow was confined to the period during which the tracing was actually falling and to the earliest portion of the ensuing interval of relatively constant oxygen concentration. The remainder of this period of constant concentration represents the passage of fluid which was between the tissue and the oxygen electrode at the instant the slow rate of flow was restored. If this fluid or the wall of the tubing was contaminated, the consequent removal of oxygen was signalled by a rise which began in the record as soon as the flow was slowed.

Survival at rest. With the nerve cells at rest under the conditions of these experiments, oxygen was consumed at a rate which usually declined very slowly. The capacity to respond to occasional stimulation of the preganglionic nerve was also retained for many hours, although the amplitude of response usually declined sooner than under certain other experimental conditions (Fig. 4: cf. LARRABEE and BRONK, 1952).

Diffusion limitations. An important technical consideration in any experiment on excised tissue is the adequacy of supply and removal of substances concerned in metabolism; these must be exchanged with the surrounding fluid by processes of diffusion. The question of oxygen supply is particularly important, because of the relatively high rate of utilization of oxygen and the limited amount dissolved in aqueous bathing solutions.

Application of formulae based on principles of diffusion (HÖBER, 1945) suggested that the concentration of oxygen in the bathing fluid was probably adequate to prevent anoxia of the core of the tissue. However these calculations utilized estimates of the diffusion coefficient for oxygen and of the shape and size of the ganglion which could not be regarded as altogether accurate. Therefore experimental tests of the adequacy of oxygen supply were made by measuring the rate of oxygen consumption with various concentrations of oxygen in the equilibrating gas. With each preparation, control measurements were made in solution equilibrated with 95 per cent oxygen, before and after the measurements of rates with lower percentages of oxygen. The latter rates were expressed as a percentage of the control rate, with interpolation when necessary between initial and final controls. The results of these experiments with varying oxygen

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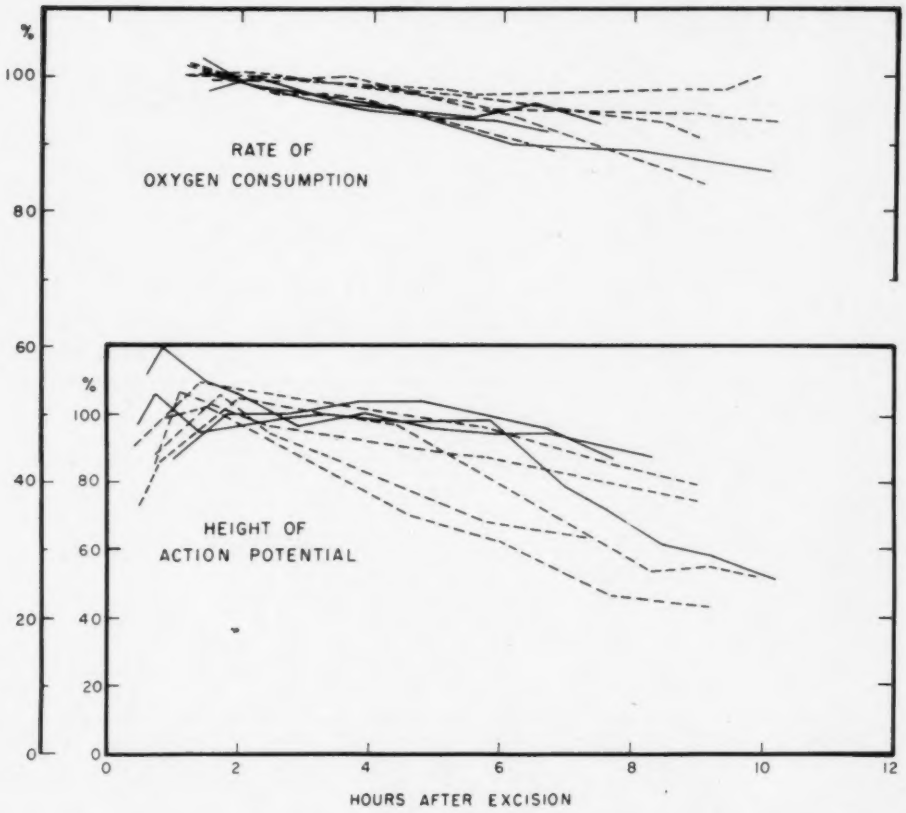

FIG. 4.—Oxygen uptake and postganglionic response to preganglionic stimulation in ganglia kept in the respirometer at 36°C. Solid lines represent preparations kept in solution equilibrated with 95 per cent O_2, 5 per cent CO_2. Broken lines represent preparations exposed to reduced oxygen tension during part of the experiment.

concentration are shown in Fig. 5. Each solid line represents several observations on a single preparation.

For purposes of comparison the changes in rate to be expected in a hypothetical sphere of metabolizing tissue is shown as a dotted curve in the figure under discussion. In calculating this curve it was assumed that initially, in solutions equilibrated with 95 per cent oxygen, the metabolic rate was uniform throughout the sphere, but that the size was 'critical,' so that the oxygen concentration fell just to zero at the centre. At lower concentrations in the solution it was assumed that the metabolic rate was uniform down to a depth at which the concentration reached zero, no oxygen of course being consumed at greater depths. This assumption is justified by evidence that oxygen consumption is independent of oxygen concentration down to very low levels in many kinds of cells, including brain tissue (ELLIOTT and HENRY, 1946), peripheral nerves (CARLSON *et al.*, 1950), and frog muscles (HILL, 1948). On the basis of these assumptions, the curve shown in the figure can be rigorously calculated. (See Appendix.) This curve cannot be expected to fit precisely the results on a ganglion, one important reason being that such a preparation is far from being a perfect

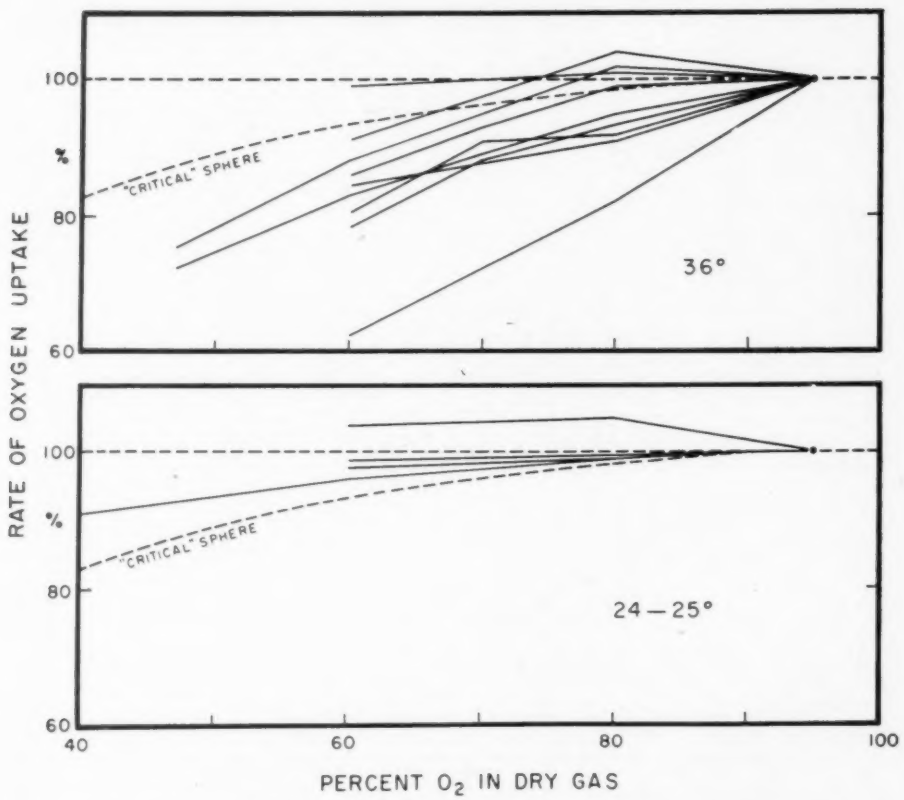

FIG. 5.—Changes in rate of oxygen consumption caused by altering the percentage of oxygen in the gas with which the bathing solution was equilibrated. Percentage of oxygen was reduced by replacement with nitrogen; all gas mixtures contained 5 per cent CO_2. Solid lines connect observations on the same preparation. The broken curve refers to a hypothetical sphere of tissue, in which the oxygen tension is assumed to be zero at centre but finite elsewhere with 95 per cent O_2, as discussed in text and Appendix. Preparations represented in upper portion of the figure were studied at 36°C, those below at 24–25°C.

sphere. But this and other calculations have proved useful for reference and particularly for emphasizing that oxygen uptake may decline very gradually as anoxia develops with decreasing external oxygen concentration. The curve is only slightly altered if it is assumed that oxygen uptake stops at any arbitrary low partial pressure in the tissue other than zero, such for example as at 1/10 of an atmosphere.

From the upper part of Fig. 5 it is apparent that at 36° the oxygen uptakes of several preparations were not significantly reduced by lowering the concentration of oxygen in the equilibrating gas from 95 per cent to 80 per cent. Accordingly oxygen utilization could not have been limited by diffusion when 80 per cent O_2 or above was used in these particular preparations. The oxygen uptake by other preparations was definitely reduced when 80 per cent O_2 was used. This might possibly be interpreted as evidence that oxygen uptake varies with oxygen concentration in sympathetic ganglion cells. However, in view of the relatively constant consumption over this range of concentrations in some preparations, together with evidence of other investigators

cited above that oxygen uptake by various tissues is independent of oxygen concentration down to very low levels, it is more reasonable to interpret these results as evidence that an anoxic core can develop in some ganglion preparations. It is suspected that this can occur even with 95 per cent O_2. This is suggested by the fact that oxygen uptake declined considerably more with decreasing oxygen concentration in some preparations than in the hypothetical 'critical' sphere. Accordingly it will be necessary to examine with care each of the experimental findings to be reported in this paper, in order to be sure that they are not misinterpreted because of the occasional presence of an anoxic core.

A possible way of avoiding the anoxic core is to reduce the temperature. This increases the concentration of oxygen in solution due to an increase in solubility, but retards oxygen movement by lowering the diffusion coefficient: these two effects tend to offset one another in determining the anoxic core. The main advantage to be expected results from a slowing of the oxygen consumption. The lower part of Fig. 5 indicates that the anoxic core is indeed removed by dropping to 24–25°, since at these temperatures there was no significant change in uptake when oxygen concentration was lowered, even down to 60 per cent. Accordingly observations at reduced temperatures will be reported in many of the following experiments.

RESULTS

Resting rate of oxygen consumption. Resting rates of oxygen uptake by a number of ganglion preparations are given in Table 1, along with the average values in each of the three temperature ranges investigated.

The mean rate stated for 36° is perhaps slightly low, owing to the possible presence of an anoxic core in some preparations, as indicated in the preceding section. The following argument indicates that the error from this source is not very great:

Experiments shown in Fig. 5 indicate that at 36° about half the preparations were free of anoxic cores at 95 per cent O_2. Let it be conservatively assumed that in fact only 25 per cent, or 6 out of the 23 ganglia investigated at 35–36°, were free of anoxia. Although it is impossible to identify these particular preparations among the tabulated results, it is obvious that their average rate of oxygen consumption can be no higher than the average of the 6 highest rates in the table. This average is equal to 355 μmole O_2/g/hr, only 12 per cent above the average for all 23 preparations. Actually the error due to anoxia is almost certainly less than this, since the percentage of non-anoxic preparations is apparently greater then 25 per cent, and these preparations probably do not all fall at the top of the rank order of rates. Thus it may be concluded that the average rate stated in Part A of Table 1 has not been diminished by as much as 12 per cent due to anoxic cores. Probably the error is actually smaller than this.

Additional evidence that anoxia had no important effect on the average resting rate is found in the observation that the average rate was the same for the 11 ganglia in Table 1 measured with the solution flowing at a rate of 0·75 ml/hr as for the 12 measured with a flow of 0·30 ml/hr. The average concentration of oxygen at the surface of a ganglion obviously increases with the rate of flow.

The average rate of oxygen uptake is compared with that of other excised rat tissues in Table 2. Ganglia occupy an intermediate position, consuming oxygen less rapidly than cerebral cortex, for example, but more rapidly than peripheral nerve trunks. The ganglion rate is in fact comparable to that which has been reported for resting diaphragm muscle.

The temperature coefficient was similar to those found by others in rat cerebral cortex and liver, although less than that reported for rat nerve trunks (Table 3).

Oxygen consumption during activity. As in other nervous tissues, oxygen was

TABLE 1.—RESTING RATES OF OXYGEN UPTAKE BY SUPERIOR CERVICAL GANGLIA OF RATS

Experiment		Animal		Conditions		Tissue	
Number	Date	Sex	Body wt. in kg	Flow rate ml/hr	Temp. °C	Dry wt. in mg	Oxygen uptake μmole/g/hr (dry wt.)
			A. Measured at 36°C				
1	22/7/54	M	0·24	0·30	36	0·26	263
2	11/8/54	F	0·18	0·30	36	0·20	284
3	15/9/54	F	0·15	0·30	36	0·14	284
4	16/9/54	F	0·20	0·30	36	0·23	297
5	21/9/54	F	0·20	0·30	36	0·23	299
6	23/9/54	F	0·19	0·30	36	0·28	233
7	3/12/54	F	0·19	0·75	36	0·24	265
8	7/12/54	M	0·19	0·75	36	0·25	304
9	29/12/54	M	0·22	0·75	36	0·20	344
10	4/1/55	F	0·22	0·75	36	0·24	249
11	5/1/55	M	0·21	0·75	36	0·20	363
12	8/1/55	M	0·21	0·75	36	0·23	284
13	13/1/55	M	0·20	0·75	36	0·20	262
14	8/4/55	M	0·19	0·30	36	0·20	313
15	9/12/55	M	0·19	0·30	36	0·19	372
16	13/12/55	M	0·19	0·30	36	0·19	331
17	15/12/55	M	0·20	0·30	36	0·21	350
18	23/1/56	F	0·19	0·30	36	0·23	272
19	6/2/56	M	0·19	0·75	36	0·19	344
20	9/2/56	F	0·18	0·75	36	0·23	303
21	17/2/56	M	0·18	0·75	36	0·32	226
22	3/3/56	M	0·20	0·75	36	0·17	356
23	6/3/56	F	0·19	0·75	36	0·26	298
			Mean rate ± S.E.M. = 300 ± 9 μmole/g/hr.				
			B. Measured at 28–30°C				
1	20/2/56	M	0·19	0·75	28	0·19	188
2	21/2/56		0·19	0·75	30	0·19	205
3	27/2/56	F	0·19	0·75	30	0·22	195
			Mean rate = 196 μmole/g/hr.				
			C. Measured at 23–25°C				
1	29/2/56	M	0·19	0·75	25	0·19	144
2	30/3/56	M	0·19	0·30	24	0·20	121
3	31/3/56a	F	0·19	0·30	24	0·19	121
4	31/3/56b	F	0·19	0·30	24	0·20	105
5	12/4/56	M	0·20	0·30	24	0·16	147
6	23/4/56	M	0·22	0·30	24	0·25	110
7	19/1/57	F	0·22	0·30	23	0·26	75
8	22/1/57	F	0·22	0·30	23	0·29	76
9	23/1/57	M	0·20	0·30	23	0·24	95
10	24/1/57	M	0·21	0·30	23	0·20	114
			Mean rate ± S.E.M. = 111 ± 8 μmole/g/hr.				

(Most of the above measurements were made between 1 and 3 hr after excision)

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TABLE 2.—RATES OF OXYGEN CONSUMPTION BY VARIOUS RAT TISSUES IN BICARBONATE-BUFFERED SOLUTIONS CONTAINING GLUCOSE
(At or near normal body temperature)

Tissue	μmole/g/hr (dry wt.)	Source
Retina	1250	ELLIOTT, 1955.
Cerebral cortex (slices)	500	ELLIOTT, 1955.
Diaphragm muscle	335	ELLIOTT, 1955.
Sympathetic ganglia	300	This paper, Table 1.
Pancreas	180	ELLIOTT, 1955.
Various nerve trunks	30–90	LARRABEE and BRONK, 1952. LARRABEE, unpublished.

TABLE 3.—TEMPERATURE COEFFICIENTS OF RATES OF OXYGEN CONSUMPTION BY RAT TISSUES

Tissue	Q_{10}	Temp. range	Source
Sympathetic ganglia	2·3	24–36°C	This paper, Table 1*
Cerebral cortex (slices)	2·1	10–35°C	FIELD, FUHRMAN, and MARTIN, 1944.
Peripheral nerve trunks	2·5	15–37°C	CRANEFIELD, 1952.
Liver (slices)	2·0	25–35°C	FUHRMAN and FIELD, 1945.

* In addition the temperature was varied during the course of two experiments, yielding values for Q_{10} of 2·4 and 2·55 respectively.

consumed more rapidly by ganglia during activity than when the cells were at rest. In experiments at 36° the increased rate of consumption remained approximately constant for as long as 2 hr during stimulation of the preganglionic nerve at frequencies of 6 per sec or lower, and promptly returned to the resting level when stimulation was stopped (Fig. 6). At 15 per sec or higher, the rate of uptake usually declined progressively after reaching an initial peak (Fig. 6*B*). Progressive failure of some of the ganglion cells to respond to each volley of preganglionic impulses may have been an important factor in this decline, since the heights of the successive postganglionic action potentials also declined at these higher frequencies. This explanation is in accord with the conclusion, reached in an earlier paper concerned with perfused sympathetic ganglia of cats, that the observed increase in ganglionic oxygen consumption caused by activity occurred largely if not exclusively in some postsynaptic structure (LARRABEE and BRONK, 1952).

The speed of transition from one rate of oxygen uptake to another cannot be determined accurately from the records reproduced here. The metabolic change in the tissue occurred so rapidly that the record probably was much distorted, at least during the initial portion of the transition. This distortion was due partly to the time required for establishment of a new steady-state of oxygen diffusion in the tissue and surrounding fluid, partly to the fact that different lengths of time were required for elements of fluid, in contact with different parts of the tissue at the time of the metabolic change, to reach the oxygen electrode (CARLSON *et al.*, 1950), and partly to

other factors. Nevertheless it is possible to set an upper limit to the actual time required for the change in rate, since the causes of distortion all tend to slow the recorded transitions. Therefore the speed of return to the resting level was measured on several records following cessation of stimulation at frequencies of 5–15 per sec, assuming that the recovery was exponential as described by BRINK, BRONK, CARLSON

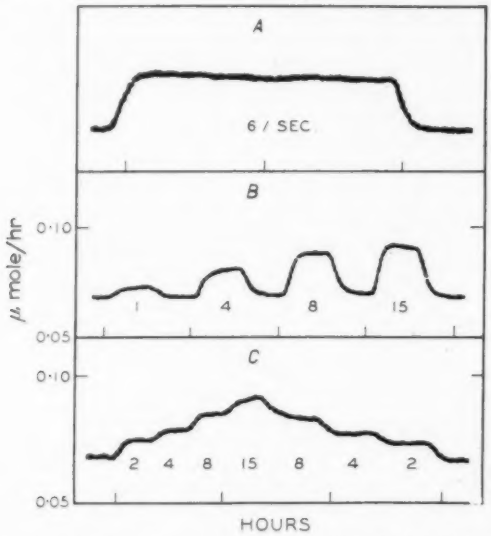

FIG. 6.—*A* Record of increase in rate of oxygen uptake during prolonged stimulation at a frequency of 6/sec. *B* Increase in rate of oxygen consumption at various frequencies. *C* Increase in rate of oxygen uptake during stimulation with step-wise increase and decrease in frequency. All at 36°c.

and CONNELLY (1952) for frog nerve. In this way time constants of recovery were found which varied from 1·7 to 2·4 min (av. 2·1 min) in 7 records at 36° and from 1·4 to 3·4 min (av. 2·1 min) in 5 records at 23°. Accordingly, the recovery in rate of oxygen consumption must have occurred with a time constant no greater than about 2 min at both temperatures, and perhaps was actually faster due to the technical limitations in the method.

In Table 4, the speed of recovery is compared with some values previously reported. Oxygen consumption returned to the resting rate much more quickly in mammalian sympathetic ganglia than in frog nerves. It may be emphasized that the difference is not simply a result of difference between the temperatures at which the two preparations were studied, since the contrast persisted when observations were made at comparable temperatures. Possible influences of other experimental variables have not yet been investigated. For example, magnesium and phosphate were included in the bathing fluid for ganglia but not for frog nerve (BRINK, personal communication; CARLSON *et al.*, 1950). Discrepancies in pH were evidently unimportant, since recovery in ganglia at 23° was not significantly altered by changing pH from 7·3 to 6·9. Thus there may be characteristic differences between the rapidity with which rates of metabolism in various nervous tissues recover following activity. One might speculate that the quicker return to resting rate represents an adaptation in a higher organism either for metabolic economy or for earlier readiness for renewed action.

TABLE 4.—TIME CONSTANTS OF RETURN TO RESTING RATE OF OXYGEN UPTAKE AFTER STOPPING REPETITIVE STIMULATION

Tissue	Species	Temp.	Time constant	References
Sup. cerv. gang.	Rat	36°	≦2 min	This paper
Sup. cerv. gang.	Rat	23°	≦2 min	This paper
Sup. cerv. gang.	Rabbit	26°	4 min	FITZHUGH and LARRABEE, 1950.
Myelinated fibres of periph. nerve	Frog	20°	18±3 min	BRINK *et al.*, 1952.

Rate of oxygen consumption as function of frequency. In order to investigate the relation between rate of oxygen uptake and the frequency of nervous action, a procedure was adopted which permitted a thorough frequency study to be made rapidly while the preparation was still relatively fresh.

Stimulation was started at a low frequency (1 or 2/sec). The frequency was then increased, usually at intervals of 10 to 15 min, up to a maximum of 15 or 30/sec, and then usually decreased at similar intervals until resting conditions were finally restored. This resulted in a record of oxygen consumption which rose and fell in steps (Fig. 6*C*). The height of each step was measured above the interpolated level of resting oxygen uptake. In experiments where measurements were made with both an ascending and a descending series of frequencies, the two results at a given frequency were averaged. Since these two measurements never disagreed by more than 21 μmole O_2/g/hr, it may be assumed that the values obtained were close to the steady-state values, with possible exceptions at frequencies of 15/sec and high. In cases where the rate of uptake fell from its initial peak at these higher frequencies, the rate was measured 10 min after the change in frequency. Throughout the period of stimulation the heights of the action potentials were measured alternately at two different intensities of stimulation, to be sure that the intensity was kept supramaximal.

Oxygen consumption was accelerated, even at very low frequencies, and rose progressively as the rapidity of stimulation was increased (Fig. 7). A maximum increase was reached at about 15/sec, above which there was no further rise. The average increase at 15/sec in 7 preparations at 36° was 42 per cent above the resting level. These results are similar to those previously obtained on excised cat and rabbit ganglia (LARRABEE and BRONK, 1952), except that the maximum percentage increase is somewhat higher in the present experiments.

It is a pertinent question why the rate of oxygen consumption was limited, instead of continuing to rise with increasing frequency of stimulation. One factor which undoubtedly contributed was an inability of the neurons to conduct or discharge impulses in very rapid succession. For example, it was indicated in the previous section of this paper that the failure of some ganglion cells to respond to every preganglionic volley may have reduced the oxygen consumption during prolonged stimulation at frequencies of 15/sec. However, evidence was found for an additional factor, possibly more significant. Even at frequencies of 8/sec and below, where there was no reduction in height of the postganglionic action potential with increasing frequency, successive increases in rate of stimulation nevertheless failed to cause proportional increases in the rate of oxygen uptake. This is shown by the decreasing slope of the oxygen curves in Fig. 7. Moreover, nearly doubling the frequency, from 8 to 15/sec, caused but little acceleration of oxygen uptake, even though the height of

the electrical response was only slightly reduced. Thus the amount of oxygen consumed per volley of impulse declined as the frequency increased, out of all proportion to the decrease in number of nerve impulses participating in the volleys. A similar effect has been described for frog nerve by BRINK, *et al.* (1952).

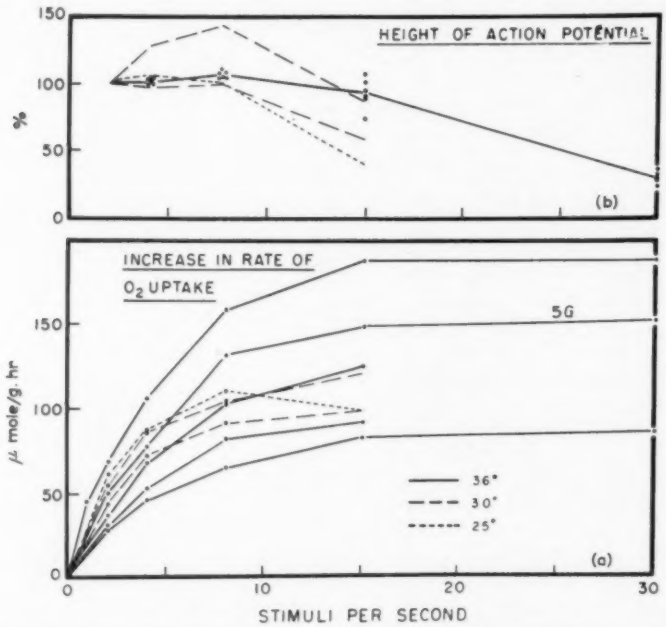

FIG. 7.—(*a*) Increase in rate of oxygen uptake above the resting rate in several preparations during maximal preganglionic stimulation. (*b*) Height of the postganglionic action potentials simultaneously observed in the same preparations, relative to the height at 2/sec. Solid lines refer to preparations studied at 36°C, others as indicated. Experiment marked 5*G* was conducted with solution containing 5 times the usual amount of glucose.

It may next be shown that the reduction, with increasing frequency, in the amount of oxygen consumed per volley is not due to a limit in the rate at which oxygen can diffuse into a ganglion. This conclusion is supported both by theoretical considerations and by experimental evidence. (a) In the first place, application of the simple diffusion theory for a hypothetical sphere of tissue, mentioned above and developed in the Appendix, indicates that the presence of an anoxic core might reduce the amount of oxygen consumed per volley at all frequencies, but would not seriously distort the *shape* of the curve relating oxygen uptake to frequency unless the anoxic core were very large indeed. (b) Secondly, all curves of oxygen uptake versus frequency at 36° are of the same form in Fig. 7, whereas evidence has already been presented that the size of the anoxic core, which is a measure of diffusion limitations, varies considerably from one preparation to another. (c) Curves obtained at reduced temperature, where the oxygen supply was certainly adequate were essentially similar to those at 36° as shown in Fig. 7. (d) Finally, two preparations were studied at three different oxygen concentrations (Fig. 8). The forms of the curves with 95 per cent and with 80 per cent O_2 were indistinguishable from one another, and even with 60 per cent O_2 the distortion was slight in one case, although greater in the other. It is true that the

absolute magnitude of the metabolic increments were somewhat reduced by lowering the oxygen concentration in these two experiments, but as shown in the figure the shapes of the curves were not significantly or consistently altered; and it is the shape rather than the magnitude which is relevant in present context.

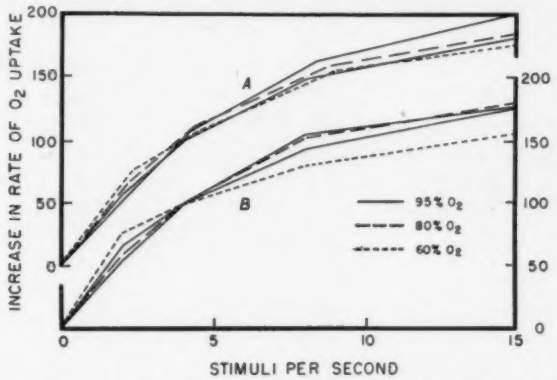

FIG. 8.—Increase in rate of oxygen uptake during maximal stimulation in the presence of various concentrations of oxygen. Increases are expressed as a percentage of the increase at 4/sec. *A* and *B* refer to two different preparations, each at 36°C. Ordinate scale for *A* on left, for *B* on right. Solid lines show increases at 95 per cent O_2 before and after the measurements at lower O_2 concentrations.

Not only was oxygen supply not a factor in shaping the relationship between oxygen uptake and frequency, but diffusion of glucose was also not restrictive. The curve marked 5*G* in Fig. 7, which was determined in the presence of 5 times the usual concentration of glucose, has the same form as all the others.

It is therefore concluded that a fundamental property of neuronal metabolism is a decrease in amount of oxygen consumed per individual nerve impulse as the frequency of action is increased and as the interval allowed for recovery between successive impulses is thereby decreased. This is in agreement with the results of BRINK *et al.* (1952) on frog nerves. Indeed the present investigation of this question was stimulated by their prior finding, which is here shown to apply to another species and to a region containing nerve cell bodies and synapses as well as nerve fibres.

DISCUSSION

Comparison of a frequency parameter with that of frog nerve. Comparison of the present results on rat ganglia with those previously reported for myelinated fibres in frog nerves (BRINK *et al.*, 1952), has shown that the curve relating increase in rate of oxygen uptake to frequency of action is similar in form for these two very different nervous tissues. However, the frequency parameter of the curve, while relatively reproducible for either preparation, differs considerably in magnitude between them. This parameter may therefore be a significant characteristic of the neuronal metabolic system.

The frequency parameter was determined by fitting the ganglion data with a curve defined by the following formula, which had previously been used by BRINK *et al.* (1952) to fit data from frog nerve:

$$\Delta_f = \Delta_\infty(1 - e^{-f/k}) \qquad (1)$$

where Δ_f = the increase in rate of oxygen consumption above the resting level at frequency f, and Δ_∞ is the asymptote approached at infinite frequency. k is the frequency parameter to be evaluated.

Before fitting the curve, the increase in oxygen uptake in each of 6 experiments at 36° was first expressed as per cent of the increase observed at 15/sec and the results averaged for each frequency. The formula was then fitted by trial and error to the mean values, yielding a value of 5·2/sec for k. The curve calculated for $k = 5{\cdot}2$/sec is shown in Fig. 9. The extent to which this curve fitted the individual observations

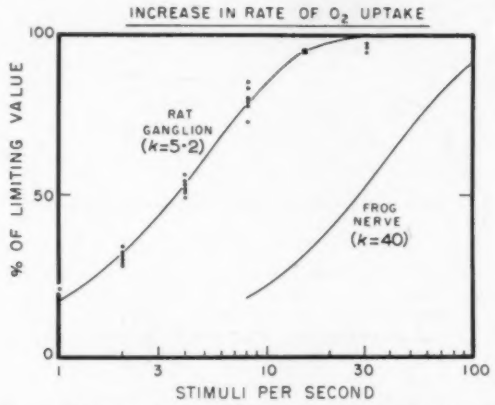

FIG. 9.—Comparison of increase in rate of oxygen uptake as function of frequency in rat ganglia (36°) and in myelinated fibres of frog nerves (20°C). Curves were calculated from the formula, $\Delta_f/\Delta_\infty = 1 - e^{-f/k}$ using the values of k indicated in reciprocal seconds. The data from ganglia were adjusted so that the increase at 15/sec in each of 6 experiments fell on the left-hand curve. Right-hand curve was reported to fit data from frog nerve by BRINK *et al.* (1952).

was demonstrated by multiplying the data from each experiment by an appropriate factor, chosen so as to make the increase in rate of oxygen consumption in that experiment fall on the curve at 15/sec. Agreement was satisfactory, except at 30/sec, where adequate data was not obtained owing to failure of some cells to respond to each stimulus (Fig. 9).

For comparison with results on rat ganglion, Fig. 9 also shows the similar curve which has been reported to fit observations on large myelinated fibres in frog nerves (BRINK *et al.*, 1952). This illustrates the difference in the frequency characteristics of the two tissues: a given fraction of the maximum metabolic response occurred in frog nerve fibres at a frequency about 8 times higher than in the rat ganglion. Half the maximum increase, for example, was reached at about 4/sec in the rat ganglion, and about 30/sec in the frog nerve.

The metabolic differences thus revealed may explain why myelinated fibres in the frog can conduct impulses at frequencies much higher than those possible in sympathetic ganglion cells or sympathetic nerve fibres. Such differences would indeed seem well adapted to the physiological functions of the two systems, since the natural frequency of discharge is typically higher in myelinated fibres of peripheral nerves than in the sympathetic nervous system. Ability to make appropriate metabolic adjustment may be advantageous throughout the entire frequency range of a neuron's physiological activity. Such metabolic adjustments, however, although normally

indicated by changes in rate of oxygen consumption, are not always so evident, at least not under non-physiological conditions. For example, the acceleration of oxygen uptake in frog nerve can be prevented by application of azide without abolishing the repetitive conduction of impulses (BRINK *et al.*, 1952).

Recovery curves for quantity of oxygen consumed per volley. If neurons returned completely to the resting state between successive responses, there would be no change in the amount of oxygen consumed per volley of impulses when the frequency of stimulation was altered. Thus the changes in the amount consumed per volley, which

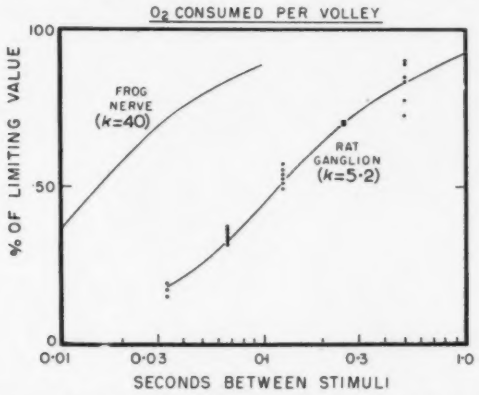

FIG. 10.—Comparison of amounts of oxygen consumed per volley as functions of the interval between stimuli in rat ganglia (36°C) and in myelinated fibres of frog nerves (20°C). Curves were calculated from the formula: $Q_f/Q_0 = (1 - e^{-f/k})/(f/k)$ for the values of k indicated in reciprocal seconds. The data from ganglia were adjusted so that the amount consumed per volley with intervals of 0·25 sec in each of 6 experiments fell on the right-hand curve. Left-hand curve was reported to fit data from frog nerve by BRINK *et al.* (1952).

have been described in this paper, demonstrate that recovery in magnitude of metabolic response is indeed incomplete during continual stimulation, even at relatively low frequencies. Curves might conceivably be constructed which would define the time course of this recovery, analogous to the familiar curves for restoration of irritability, conduction velocity, height of action potential, etc. Such recovery curves are commonly traced by recording the response to a single stimulus at various times after the end of a period of activity: unfortunately methods are not yet adequate for making such measurements on the oxidative response to an individual stimulus. However, some evidence concerning relative rates of recovery in different tissues can be obtained from steady states of continual stimulation.

The amount of oxygen consumed per volley can obviously be calculated by dividing the steady-state rate of consumption by the frequency of stimulation. The relationship between amount and frequency can also be derived from equation (1), by dividing both sides by the frequency. It can thus be shown that

$$Q_f = Q_0(1 - e^{-f/k})/(f/k) \qquad (2)$$

where Q_f = quantity of oxygen consumed per volley at frequency f, Q_0 = the limiting quantity per volley approached at zero frequency, and k is the same frequency parameter as in equation (1). Using $k = 5{\cdot}2$/sec as determined in the previous section, the recovery curve was calculated by equation (2) and is shown in Fig. 10. The interval

between stimuli, rather than the frequency, was chosen as abscissa, in order to express the relationship in terms analogous to a recovery curve. Satisfactory agreement with the individual observations is also demonstrated in the figure. For this purpose the results from each experiment were multiplied by an appropriate factor, chosen so as to

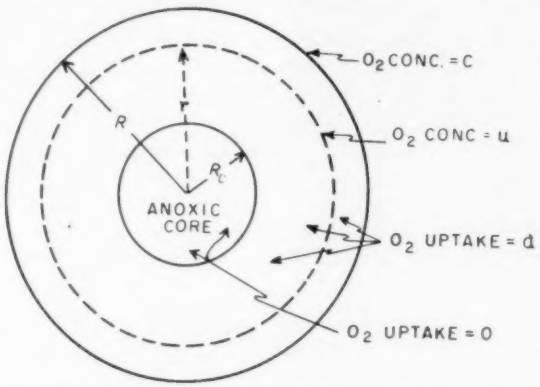

FIG. 11.—Hypothetical metabolizing sphere with anoxic core.

make the amount consumed per volley at an interval of 0·25 sec fall on the calculated curve. Also shown is the relationship calculated from the constant given by BRINK *et al.* (1952) for frog nerves.

The results illustrate a difference between the rat and frog tissues. For example, 50 per cent of the maximum responses per volley required intervals between stimuli greater than 0·1 sec in the rat ganglia, but occurred with intervals of less than 0·02 sec in the frog nerve. Thus recovery measured in this way was slower in the ganglion, despite the higher temperature, which was about 36° compared to 20° for the frog experiments.

It should be emphasized that the curves shown are not simple plots of the course of recovery following a constant amount of antecedent activity. At any frequency of stimulation the metabolic response evoked by a particular volley was influenced not only by the time which had elapsed since the last preceding volley but also by an interval of twice that duration since the next-to-the-last volley, and so on. Consequently the simpler recovery curve for a single volley placed at various times after a fixed train of stimuli, if it could be determined, would presumably rise less steeply than that shown. In view of these complexities it is difficult to attach quantitative significance to the time characteristics revealed in Fig. 10. Relatively speaking, however, it is apparent that recovery of metabolic response proceeded much more slowly in rat ganglia than in frog nerves, even though the converse might have been expected because of the temperature differences.

In conclusion it may be noted that the relative rates of these metabolic recoveries differ between the two tissues in the same sense as many other time parameters; sympathetic neurons are typically the slower tissue, whether comparison be made on the basis of conduction velocity, duration of various components of the action potential, recovery of irritability, or recovery of metabolic response. By way of contrast, however, one metabolic time characteristic may be recalled in which the relative speeds of the two tissues stand in a reverse relationship to those just enumerated;

this is the speed with which the rate of oxygen uptake declines when activity is halted (Table 4). The original resting metabolic rate is then regained more quickly in the rat ganglion, despite the evidence that recovery in amount of oxygen consumed per volley is slower than in the frog nerve. Thus two metabolic recovery processes can be distinguished, although the nature of these processes and the reasons for the quantitative differences from one neuronal tissue to another remain unknown.

APPENDIX

Steady-state oxygen uptake by a hypothetical metabolizing sphere with anoxic core

Referring to Fig. 11:

R = radius of sphere
R_c = radius of anoxic core
$F = R_c/R$
u = oxygen concentration at any radius (r) between R and R_c; u is assumed equal to zero at $r \leq R_c$
C = concentration of oxygen at surface of sphere
a = oxygen consumed per unit volume, assumed uniform at all radii between R and R_c and equal to zero at $r \leq R_c$
Q = total rate of oxygen uptake divided by total volume of sphere
V = volume of sphere
D = diffusion coefficient of oxygen within the sphere

The anoxic core is thus defined as a region within which no oxygen is present and therefore no oxygen can be consumed. The assumption that uptake per unit volume ($=a$) is uniform at all radii between R_c and R is justified by evidence cited earlier in this paper indicating that oxygen uptake is independent of oxygen concentration down to very low concentrations in various tissues (ELLIOTT and HENRY, 1946; CARLSON *et al.*, 1950; HILL, 1948). The units of measurement are left undefined, except that they must obviously be consistent in using the formulae to be derived. Actually units do not enter into present applications, since all variables are expressed in relative terms in the final equations.

In a steady state the total flux of oxygen inward through a sphere of any radius (r) between R and R_c may be expressed in two ways:

(1) Inward flux = (area of sphere of radius r) × (diffusion coef. of oxygen) × (inward gradient of O_2 concentration)
$= 4\pi r^2 D(du/dr)$

(2) Inward flux = total rate of O_2 uptake inside radius r
$= (a) \times$ (non-anoxic volume inside r)
$= (a)\,(4\pi/3)\,(r^3 - R_c^3)$

Equating these two expressions for inward flux:

$$du/dr = (a/3D)(r - R_c^3/r^2)$$

Integrating and applying the boundary condition that $u = C$ at $r = R$

$$u = C - (a/3D)(R^2/2 - r^2/2 + R_c^3/R - R_c^3/r) \tag{3}$$

Since it has been assumed that $u = 0$ at $r = R_c$, the preceding equation reduces to

$$6CD/aR^2 = 1 - 3(R_c/R)^2 + 2(R_c/R)^3 \tag{4}$$

This defines the radius of the anoxic core (R_c) in terms of variables which appear on the left of the equation.

Another relation can be obtained by expressing the total rate of oxygen uptake for the whole sphere ($= QV$):

$$QV = (a) \times \text{(non-anoxic volume)} = a(4\pi/3)(R^3 - r_c^3)$$

or

$$Q = a[1 - (R_c/R)^3] \tag{5}$$

It is convenient to rewrite equations (4) and (5) in terms of the ratio (F) of the radius of the core to the radius of the whole sphere:

$$(6CD)/(aR^2) = 1 - 3F^2 + 2F^3 \tag{6}$$

$$Q = a(1 - F^3) \tag{7}$$

It may be noted that F^3 expresses the anoxic volume as a fraction of the total volume.

In determining how the total oxygen uptake of the hypothetical sphere would vary with external oxygen concentration, it is necessary to consider only relative values under conditions where D, a, and R are constant. Therefore

$$C_2/C_1 = (1 - 3F_2^2 + 2F_2^3)/(1 - 3F_1^2 + 2F_1^3) \tag{8}$$

$$Q_2/Q_1 = (1 - F_2^3)/(1 - F_1^3) \tag{9}$$

If we measure C in per cent of O_2 in the dry equilibrating gas, and consider only the 'critical' case where 95 per cent O_2 is just adequate to prevent an anoxic core, then equation (8) becomes:

$$C_2 = 95(1 - 3F_2^2 + 2F_2^3) \tag{10}$$

If we further express the total rate of oxygen uptake relative to a value of 100 at 95 per cent O_2, then equation (9) becomes:

$$Q_2 = 100(1 - F_2^3) \tag{11}$$

It is now possible to assume a series of values for F_2, and to calculate a series of corresponding values for Q_2 and C_2. Plotting Q_2 against C_2 will then show how the rate of oxygen uptake may be expected to vary with external oxygen concentration in the hypothetical 'critical' sphere. This is the relationship shown by the dotted curves in Fig. 5.

In conclusion it may be noted that equations (8) and (9) may be used to determine how Q would vary with C, starting with any given fraction of the volume anoxic at any stipulated O_2 concentration. For example, the larger the volume anoxic at 95 per cent O_2, the more rapidly does the uptake fall as oxygen concentration is lowered. Moreover, equations (6) and (7) may be manipulated to define the changes in uptake when variables other than C are altered.

SUMMARY

The rate of oxygen consumption by superior cervical ganglia excised from adult rats was measured using sterile techniques in a newly designed continuous-flow respirometer. Rate of oxygen uptake was calculated from the concentration of oxygen remaining in the solution leaving the respirometer, measured electrochemically with a polarized platinum electrode. The respirometer contained electrodes for stimulating the preganglionic nerve and for recording the action potentials of the preganglionic and postganglionic nerves. The experimental arrangement excluded the stimulated region of the preganglionic nerve from the oxygen measurements. Therefore changes in rate of oxygen uptake observed during stimulation were a consequence of conducted impulses and were not direct effects of the stimulating current.

The bathing fluid was equilibrated with 5 per cent CO_2, 95 per cent O_2, was buffered with bicarbonate, and contained glucose in addition to the usual inorganic ions. Preparations survived many hours at 36°C, usually with less than 15 per cent drop in the rate of oxygen uptake from the second to the ninth hour after excision. From measurements with various oxygen concentrations in the bathing fluid, it was concluded that some but not all preparations had an anoxic core in solution equilibrated with 95 per cent O_2 at 36°C; none were anoxic at 23–24°C.

Resting rates of oxygen consumption, in μmole/g hr (dry weight) with S.E.M. and numbers of observations, were $300 \pm 9(23)$ at 36°, and $111 \pm 8(10)$ at 24°. From these data, $Q_{10} = 2{\cdot}3$.

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Rate of oxygen uptake increased during repetitive stimulation, approaching a maximum at about 15/sec. At this frequency the increase was about 40 per cent (average of 7 preparations at 36°C). Evidence indicated that the failure of oxygen uptake to increase in proportion to frequency was not explained by a failure of impulse uptake to increase further at higher frequencies was not due to failure either of impulse conduction or of synaptic transmission, nor was it due to inability of oxygen or glucose to diffuse rapidly enough into the tissue. It is concluded that the amount of oxygen consumed per individual nerve impulse declines as the frequency of action is increased in neurons of the mammalian sympathetic nervous system, in agreement with results of BRINK *et al.* (1952) on myelinated fibres in peripheral nerves of frogs.

Results on the increase in rate of oxygen consumption at various frequencies of stimulation fitted an exponential relation previously employed by BRINK *et al.* (1952) for frog nerves, but with a different value for the frequency parameter. Thus a formal similarity was found between these two tissues. The amount of oxygen consumed per volley recovered from previous activity more slowly in the rat ganglia than in the frog nerve. This difference in a metabolic characteristic may be compared to the slower speed, in various physiological characteristics, of mammalian sympathetic neurons relative to frog myelinated nerve fibres. The direction of these differences, however, contrasted with the faster decay in rate of ganglionic compared to axonal oxygen uptake when repetitive stimulation was suddenly stopped.

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GLUCOSE CONSUMPTION AND LACTATE PRODUCTION IN A MAMMALIAN SYMPATHETIC GANGLION AT REST AND IN ACTIVITY*

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(*Received* 8 *August* 1957)

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THESE experiments are part of a study of relationships between metabolism and function in mammalian sympathetic ganglia. This study was initiated to take advantage of the long survival of synaptic transmission and axonal conduction in excised ganglia under conditions permitting well-controlled measurements of cell metabolism.

It has been shown previously that an excised ganglion, weighing as little as one milligram, consumes glucose at a measurable rate and loses its capacity for function if this necessary external supply of substrate is removed (LARRABEE and BRONK, 1952; EDWARDS and LARRABEE, 1955). Additional measurements of glucose uptake will be described in the present paper, along with determinations of lactic acid production. These observations, combined with recent measurements of oxygen consumption (LARRABEE, 1957), demonstrate an approximate quantitative agreement between the utilization of glucose and of oxygen under conditions of rest, in accord with the generally accepted contention that glucose is the major substrate for oxidative generation of energy in resting neurons. In addition it will be shown that glucose uptake, like oxygen consumption, is accelerated during activity, but that the additional glucose apparently is not oxidized. These changes during activity, which have been studied at a temperature of 36° in the experiments to be reported here, differ in some ways from those found more recently in experiments at 23°, which will be described in a subsequent paper (DOLIVO and LARRABEE, 1957*a*,*b*,*c*).

METHODS

Superior cervical ganglia, together with 10 to 15 mm lengths of preganglionic nerve and 2 to 3 mm lengths of post-ganglionic nerve, were excised from adult hooded rats of both sexes (Lashley strain) with body weights usually between 180 and 220 g. Details of anaesthesia with intraperitoneal urethane, determination of dry weight of tissue, typical weights found, etc., were as stated by LARRABEE (1957). The capacity for conduction of impulses along the nerve trunks and for synaptic transmission through the ganglion was maintained throughout most of the experiments. The experiments were conducted in a temperature-controlled box at about 36°.

Bathing solution. The bathing solution was made sterile as described elsewhere (LARRABEE, 1957), giving final concentrations as follows in m-moles per litre: NaCl, 136; KCl, 5·6; $CaCl_2$, 2·22; $MgCl_2$, 1·2; $NaHCO_3$, 16·2; NaH_2PO_4, 1·2; glucose 5·5. Equilibration with 5% CO_2, 95% O_2 gave a pH of 7·3 to 7·4.

* Supported in part by a research grant (No. B–702) from the Institute of Neurological Diseases and Blindness of the National Institute of Health, United States Public Health Service. Preliminary observations were aided by a grant from the Supreme Council, Scottish Rite Masons, for research under the direction of D. W. BRONK. Preliminary reports have been published by HOROWICZ (1955), LARRABEE and HOROWICZ (1955, 1956), DOLIVO *et al.* (1956), and LARRABEE *et al.* (1957).

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Collection and analysis of samples. The preparation was supported in a small chamber through which there was a flow of moistened gas mixture containing 95% O_2, 5% CO_2 (Fig. 1). A small glass vessel containing bathing fluid was raised and lowered in the moist chamber by a motor-driven lever system, so that the ganglion was dipped into the solution for about 15 sec of each minute, in order to supply glucose and remove lactate. The volume of fluid was kept small—approximately 30 μl—so that about 10 per cent of the glucose was removed in one hour. The fluid was reproducibly measured with a Linderstrøm-Lang-Holter constriction pipette. At the end of one or two hours the vessel was replaced with one containing fresh solution. The contents of each vessel were rinsed into a test tube with 1·8 ml of distilled water. This was covered and stored in a refrigerator. In addition the contents of two control vessels which had not been exposed to the tissue were similarly saved from each experiment.

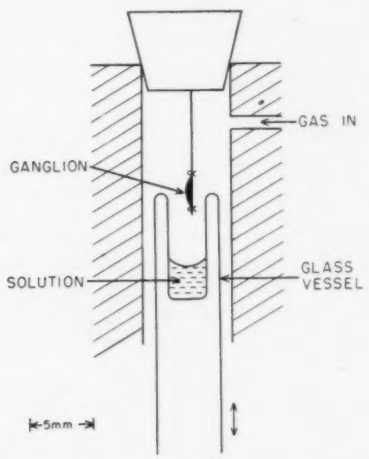

FIG. 1.—Arrangement for dipping a ganglion in about 30 μl of bathing solution for about 15 sec of each minute. The glass vessel was carried in a moving plastic sleeve (not shown), which prevented air from diffusing to the preparations from below. Moistened 95% O_2, 5% CO_2 entered near the top, as shown (EDWARDS and LARRABEE, 1955).

The next day, or at most two days later, three 0·5 ml aliquots were withdrawn from the 1·8 ml in each test tube, two to be analysed for glucose by a modification of the method of PARK and JOHNSON (1949) and one to be analysed for lactate by a modification of the method of BARKER and SUMMERSON (1941). The standard deviation of an analysis of an aliquot was 0·11 μg for glucose and 0·05 μg for lactate. Methods of estimating these deviations and certain details of the analytical procedures are given in the Appendix.

In making the chemical analyses the preliminary precipitation of protein, which is usually necessary in analysis of blood or other tissues, was omitted. This was done because there was no reason to expect significant amounts of protein to appear in fluid bathing a surviving ganglion with intact connective sheath.

Stimulation and recording of action potentials. The ganglion was supported in the moist chamber on a small assembly of five electrodes, made of platinum wires 0·008 in. in diameter, imbedded in lead glass except for tiny loops at the ends (Fig. 2). In order to obtain adequate drainage from this electrode assembly following each immersion in the bathing fluid, it was found necessary to provide a hydrophobic coating of Desicote, which was renewed before each experiment. With the good drainage thus provided, action potentials could be recorded, usually quite free of stimulus artifact, except during the fraction of each minute when the preparation was immersed in the fluid (Fig. 3).

The preganglionic nerve was stimulated by brief shocks of variable strength and duration which were led to the electrodes through an isolating transformer. By varying the strength of stimulus while watching the height of the postganglionic action potential, the strength was set at a supra-maximal value and was readjusted whenever necessary during the course of an experiment. The

action potential of the postganglionic nerve was fed into a capacity-coupled amplifier and either measured directly on the face of a cathode ray tube or photographed.

Sterile precautions. Because the results were to be compared with determinations of rate of oxygen consumption which were conducted aseptically, and because it had been found that contamination by micro-organisms could contribute an oxygen uptake comparable to that of the tissue itself, the present experiments were performed under conditions intended to minimize such contamination. Unfortunately it was not possible to autoclave either the electrode assembly or the moist chamber, nor to seal the chamber completely against possible additional contamination during the course of an experiment. However, the bathing solution was made sterile as previously described, and an autoclave was used to sterilize dissecting instruments, drapes, thread, sponges and the small glass vessels and pipettes used for handling the bathing fluid.

Experimental procedure and calculation of the results

Metabolic rates were calculated from differences between the analytical results on (a) control samples of bathing fluid not exposed to the tissue and (b) successive samples of fluid, each of which had been exposed to the tissue, usually for a period of one hour. Exposure of the first of these samples began about one hour after excision of the preparation. For the first two samples, the ganglion was kept at rest under standard conditions. The rates thus found were averaged in order to obtain the initial control rates. The experimental variable was introduced at the start of the third hour, and the subsequent results were expressed as changes from the control rates.

It was found, as will be described below, that rates of both glucose uptake and lactate production increased with time even when control conditions were maintained. Therefore it was necessary to compare alterations in rates which followed an experimental change with alterations which occurred spontaneously over the same time interval under control conditions.

All results are expressed on a dry weight basis.

RESULTS

Resting rates of glucose uptake and lactate output. The average initial rates of glucose uptake and lactate production of 46 preparations during the first two hours of measurement under control conditions are given in Table 1. These rates will be discussed later in relation to oxygen data after other experiments necessary for interpretation have been presented.

TABLE 1.—INITIAL RATES OF GLUCOSE UPTAKE AND LACTATE OUTPUT BY GANGLION PREPARATIONS (INCLUDING NERVES)

Average of first two hours of measurement on 46 preparations under control conditions. Mean ± S.E.M. in mg/g/hr (dry weight). Numbers of preparations in parentheses.	
(A) Glucose uptake	11·68 ± 0·33 (46)
(B) Lactate output	3·44 ± 0·19 (46)
(C) Difference between glucose uptake and lactate output*	8·23 ± 0·41 (46)

* Average of differences in individual experiments.

A few measurements were made on portions of the preganglionic nerve trunk, approximately equal in length to those ordinarily included in the ganglion preparations. Accurate measurements of metabolic exchange were not obtained on the very small amounts of tissue that these nerves provided. The results nevertheless served to show that the preganglionic nerve trunk contributed a relatively small

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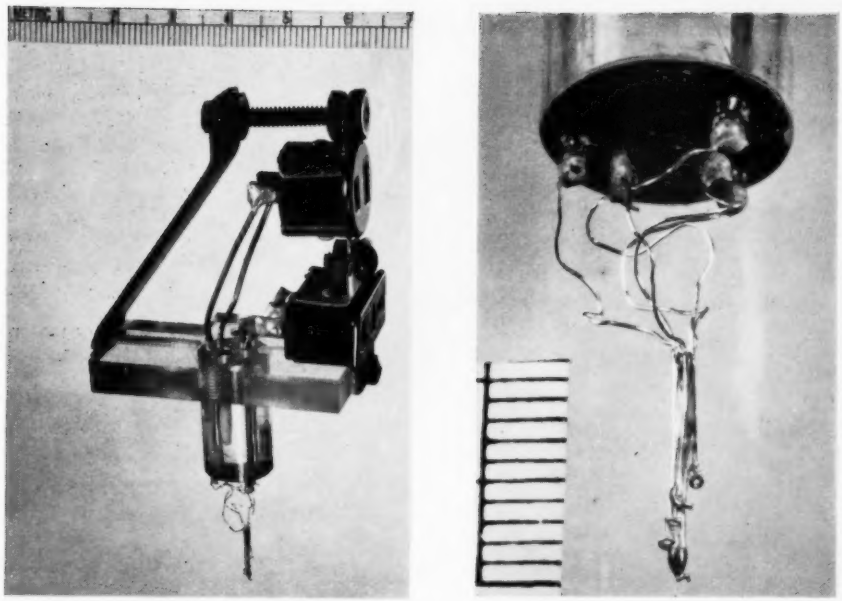

FIG. 2.—Electrode assembly which replaced the simple ganglion support shown in Fig. 1 in most of the experiments described in this paper. *Left:* The entire assembly, with scale at top numbered in centimetres. *Right:* Enlarged view of electrodes, with scale at left in millimetres. The lower two electrodes served for stimulating the preganglionic nerve, the middle one for a ground connection to the preganglionic nerve, and the upper two for recording from the postganglionic nerve. After passing the nerves through the electrode loops, the preparation was held in place by fine silk threads tied to the ends of the nerves.

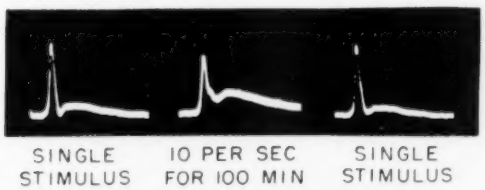

FIG. 3.—Action potentials recorded during an experiment concerned with the metabolic changes caused by repetitive stimulation. *Left:* Postganglionic response to a single supramaximal stimulus to the preganglionic nerve, about 2 hr after excision of the preparation. *Centre:* Response recorded during repetitive stimulation at a frequency of 10 per sec, 100 min after start of stimulation. *Right:* Response to a single stimulus, 40 minutes after the end of 2 hr of repetitive stimulation and nearly 6 hr after excision.

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fraction of the total metabolism in the usual preparation of ganglion plus nerves (Table 2, part A). (It should be emphasised that rates in the table are expressed in total amounts per hour, and have not been divided by the weight.) Since the length of postganglionic nerve was much less than that of the preganglionic nerve, the contribution of both must have been small. Therefore the rates reported in this paper are largely those of the ganglion, including its constituent cell bodies and nerve fibres.

TABLE 2.—COMPARISON OF METABOLIC RATES IN GANGLION PREPARATIONS (INCLUDING NERVES) WITH RATES IN PREGANGLIONIC NERVES ALONE
Mean ± S.E.M.

	(A) No stimulation				(B) Increase during stimulation (9·7 per sec)*			
	No.	Dry wt. (mg)	Resting glucose uptake (μg/hr)	Resting lactate output (μg/hr)	No.	Dry wt. (mg)	Increase in glucose uptake (μg/hr)	Increase in lactate output (μg/hr)
Ganglion with nerves	46	0·301	3·5 ± 0·1	1·0 ± 0·1	6	0·339	2·6 ± 0·5	1·8 ± 0·5
Preganglionic nerve alone	6	0·077	0·5 ± 0·1	0·4 ± 0·1	5	0·080	0·5 ± 0·2	0·3 ± 0·1

* Increase for ganglion preparations = rate during 1 hr of stimulation less average rate during preceding 2 hr at rest. Increase for nerves = rate during 2 hr of stimulation less rate during preceding 2 hr at rest. Increases were not corrected for spontaneous increase without stimulation, since this was small compared to the increase with stimulation.

The average initial rate of glucose uptake by a ganglion, measured with reducing methods for glucose analysis in the present experiments, may be compared with that found previously using a method of analysis based on an enzyme, glucose oxidase, which is much more specific for glucose (EDWARDS and LARRABEE, 1955). Because dry weight was not determined in the earlier investigation, and because we regard wet weight as unreliable for such small amounts of tissue, the comparison has to be made between rates of uptake per whole preparation rather than per unit weight. Accordingly the data must be selected from animals of comparable size. Examination of the unpublished protocols of EDWARDS and LARRABEE revealed that 12 of the 50 ganglia studied by them were from rats with body weights between 180 and 230 g and therefore comparable to the animals used in the present experiments. The average glucose uptake for these 12 ganglia was 3·4 ± 0·2 μg/hr (mean ± S.E.M.). Since these ganglion preparations did not include a significant length of preganglionic nerve, the results should be compared with the resting rate given in Table 2 after subtracting the rate indicated for the preganglionic nerve. This gives a rate for glucose uptake of 3·0 ± 0·2 μg/hr in the present experiments. Thus there was no significant difference between the values obtained using the two methods for measuring glucose.

Spontaneous changes in rates of glucose uptake and lactate output. As a basis for other experiments it was necessary to know how the metabolic rates varied with time when the tissue was kept at rest under control conditions. An investigation of this question on five ganglion preparations showed that the rates of both glucose utilization and lactate production increased as the experiments progressed. The averaged data are listed in Table 3.

TABLE 3.—SPONTANEOUS CHANGES IN METABOLIC RATES IN GANGLION PREPARATIONS (INCLUDING NERVES)

Mean ± S.E.M. ($n = 5$) in mg/g/hr (dry wt.)

Hours (Note 1)	Glucose uptake	Lactate output	Difference (Note 2)
0–1	10·2 ± 1·7	3·3 ± 0·5	7·0 ± 1·7
1–2	11·1 ± 1·0	3·5 ± 0·5	7·6 ± 0·8
2–3	12·0 ± 1·1	4·6 ± 0·7	7·4 ± 0·8
3–4	12·4 ± 1·1	5·0 ± 0·7	7·4 ± 0·9
4–5	12·7 ± 0·9	5·6 ± 0·4	7·1 ± 0·7

Note 1: Time in hours after start of metabolic measurements, which began about 1 hr after excision.

Note 2: Averages of the differences between the rates for glucose and for lactate in the individual experiments.

The final column of Table 3 lists the differences between the glucose and lactate rates. This difference did not change significantly over the time interval studied. Reasons will be given later for assuming that the lactate which is produced is formed from the glucose which is consumed. The difference between the two rates is therefore a measure of the amount of glucose which is not converted into lactate. This much glucose is accordingly available for other uses, such as oxidation. The results show that this glucose remainder did not vary significantly with time over the five hours investigated, despite the increases in the individual rates for both glucose and lactate.

Increase in rate during activity. Repetitive supramaximal stimulation of the preganglionic nerve caused considerable increase in the rates of both glucose uptake and lactate production. Fig. 4 illustrates these metabolic effects in two experiments in which activity was induced at a frequency of 15 per sec. At this, as at other frequencies, the metabolic rates rose at least as high during the first hour of stimulation as during a second hour. The rates promptly returned approximately to the resting level after the end of the activity, provided due allowance was made for the spontaneous upward drift observed in other experiments when no stimulation was applied.

A surprising finding was that the increase in lactate production was on the average almost exactly equal to the increase in rate of glucose uptake. Thus all the extra glucose evidently was put out as lactate, and the difference between glucose uptake and lactate output remained nearly constant. This implies that the amount of glucose made available for uses other than lactate production was not altered during activity.

Comparable increments in the rate of glucose uptake during stimulation were reported in 1917 for excised spinal cords of frogs by HIRSCHBERG and WINTERSTEIN

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and later for peripheral nerves of frogs by the same authors (1919). By contrast HOLMES, GERARD and SOLOMON (1930) were unable to find acceleration of carbohydrate disappearance during activity in nerve trunks from frogs or rabbits. These nerves were suspended in a gas phase and thus not provided with an external source of glucose. Results on frog nerve which might at first glance also seem to disagree with ours have been reported by MULLINS (1953). Using tracer techniques the latter author found no increase during stimulation in the yield of radioactive CO_2 from glucose tagged with ^{14}C. But it turns out that our observations are actually in

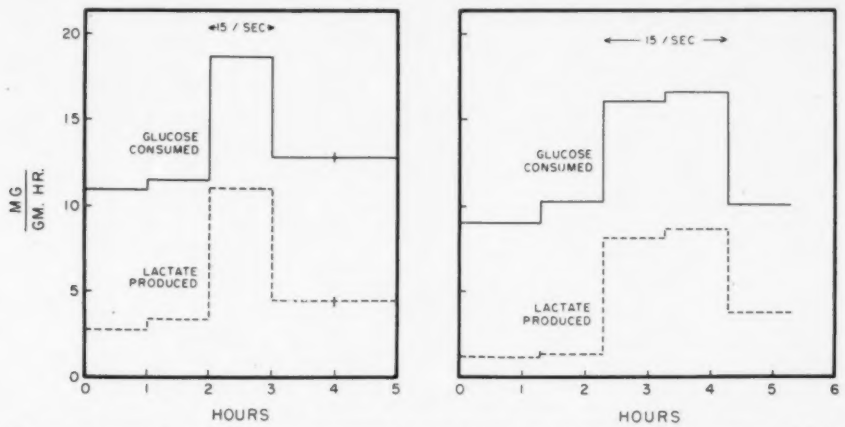

FIG. 4.—Increase in rates of glucose consumption and lactate production caused by repetitive stimulation of the preganglionic nerve during the time intervals indicated at the top. The two experiments were performed on different preparations.

agreement with those of MULLINS, since the extra glucose was apparently not oxidized in ganglia, but instead was converted to lactate.

The increments which we have observed were mostly due to altered metabolism of ganglionic constituents, since the rates at which glucose was consumed and lactate was produced changed much less during stimulation when the ganglion was cut off from the preganglionic nerve and the nerve alone was measured (Table 2, part B). It should be noted that this comparison was made on the basis of metabolic rates per whole preparation rather than per unit weight. Thus the difference was no doubt largely due to the much smaller weight of the nerve. The results also show that the metabolic changes which accompany activity in the ganglion preparations must have been largely if not exclusively associated with impulse conduction and discharge, rather than being direct effects of the electric current on the stimulated end of the preganglionic nerve. The latter effects should have been as great with the nerve alone as in a complete ganglion preparation.

Increase in rates as a function of frequency. The rates of both glucose uptake and lactate production increased with the frequency of stimulation, at least up to 15 per sec (Fig. 5). Higher frequencies were not investigated, because the height of the postganglionic response declined rapidly above 15 per sec.

In collecting the data for Fig. 5, the metabolic rates were first measured for two successive hours without stimulation, followed by one hour with stimulation. The increments in glucose and lactate rates, caused by activity in each preparation,

were calculated by subtracting the average rates for the two resting periods from the rates during the third period. The increments thus determined were added to the grand average of the resting rates measured during the first two hours on 46 preparations, already given in Table 1 and indicated by arrows at the left in Fig. 5. Thus the absolute rates during stimulation were estimated by adding the increments measured in a relatively few experiments to an average initial resting rate which was determined more accurately in a large number of experiments. This was believed to be the most effective way of manipulating the available data. In order to permit

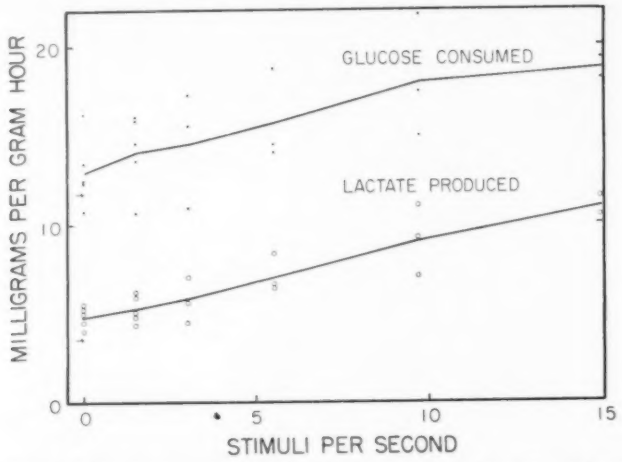

FIG. 5.—Rates of glucose consumption and lactate production as functions of frequency of neuronal activity. Repetitive supramaximal stimulation was applied to the preganglionic nerve during the third hour of the metabolic measurements. Points indicate metabolic rates in individual experiments. Lines are drawn through averages. Small arrows at left indicate average rates at rest during the first two hours. (Details concerning methods of calculation are given in the text.)

allowance for the spontaneous increase which occurred over the same time span, results of five experiments without stimulation were similarly analysed and are plotted at the abscissa corresponding to zero frequency of stimulation in Fig. 5.

It is evident from Fig. 5 that at each frequency of neuronal activity the rate of lactate production increased by approximately the same amount as did the rate of glucose uptake. The significance of this will be further considered in the Discussion.

Diffusion considerations. It is a relevant question in all studies on excised tissue whether materials drawn from the bathing fluid are adequately supplied by diffusion to cells in the centre of the metabolizing tissue. In the present instance oxygen and glucose must both enter by diffusion. In a previous paper it has been shown that at 36° oxygen is adequately supplied to some preparations of excised rat ganglia such as those used in the present studies, since the rate of oxygen consumption was not affected by moderate reduction in the external oxygen concentration. However, many other ganglion preparations did appear to have anoxic cores at 36° (LARRABEE, 1957). The oxygen supply may possibly have been even more restricted in the present experiments since the ganglia lay against the surface of the electrode assembly instead of being surrounded on all sides by solution as in the respirometer. On the other hand direct exposure to the gas phase presumably tended to improve the oxygen supply. For these various reasons, a test of the adequacy of oxygenation

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seemed desirable under the conditions employed here. This was done by seeking changes in resting rates of glucose uptake and lactate production when the oxygen concentration was lowered by substituting nitrogen for oxygen in the equilibrating gas. Carbon dioxide was kept constant at 5 per cent.

Upon complete withdrawal of oxygen, the rates for both glucose uptake and lactate production increased greatly and became equal to one another (lowest line in Table 4).

TABLE 4.—EFFECTS OF CHANGES IN OXYGEN CONCENTRATION ON METABOLIC RATES IN GANGLION PREPARATIONS (INCLUDING NERVES)

In each experiment 95% O_2 was used during the first and second hours, followed by the indicated concentration during the third and fourth hours. Rates in mg/g/hr (dry wt.).

Oxygen conc. during third and fourth hours	No.	Rates during third and fourth hours			Increase over average control rates for first and second hours	
		Glucose uptake	Lactate output	Glucose uptake minus lactate output	Glucose uptake	Lactate output
95%	5	12·2	4·8	7·4	1·5	1·4
80%	3	13·2	5·3	7·9	0·8	1·8
60%	3	14·2	8·1	6·1	1·8	2·7
0%	5	22·2	21·9	0·3	11·3	18·4

Standard errors of the above means varied from 0·4 to 2·1 mg/g/hr.

Therefore, if any anoxic core had developed when oxygen was more moderately reduced, or if an initially anoxic core had increased in size, this should have been indicated (a) by an increase in both the glucose and lactate rates, and (b) by a reduction in the difference between the two rates. Experimental test showed no statistically significant changes either in the individual rates or in the difference between them when the oxygen concentration was lowered from 95 per cent to either 80 or 60 per cent (Table 4). It is therefore assumed that there was no significant anoxic core, at least in resting preparations.

During activity the increase in rate of oxygen consumption creates a new problem, for an external oxygen concentration which was adequate at rest may then become insufficient. Therefore the question must be considered whether the increased glucose and lactate exchanges observed during activity could be the result of an anoxic core developed by the greater rate of oxygen utilization. The following evidence indicates that the activity increments can not in fact be explained by anoxia:

(1) Subsequent experiments at 23°, where respirometry revealed no anoxic cores, have also shown acceleration of glucose uptake and lactate output during activity (DOLIVO and LARRABEE, 1957*a*, *b*, *c*.)

(2) Total anoxia, induced by replacing all oxygen with nitrogen, caused an increase in glucose uptake amounting to 9·8 mg/g/hr (Table 4), while stimulation at a frequency of 15 per second, for example, caused an increase of 5·7 mg/g/hr. Therefore the increase associated with activity might

be explained by anoxia if 58 per cent of the volume were deprived of oxygen as a result of the activity. Such a large anoxic core would undoubtedly have blocked many of the synaptic pathways through the ganglion during the hour of stimulation required for the metabolic measurement and thus would have been signalled by a considerable reduction in height of the postganglionic action potential (BRONK *et al.*, 1948). Since this did not occur, anoxia must have been relatively insignificant. Accordingly the observed increase is much too great to explain by expansion of an anoxic core.

A similar argument may be based on lactate output, which increased by 17·0 mg/g/hr during total anoxia and by 6·3 mg/g/hr during stimulation at 15 per sec.

(3) According to diffusion theory (HÖBER, 1945) the oxygen concentration falls to zero at the centre of a uniformly respiring sheet, cylinder or sphere of tissue and is finite elsewhere when

$$a/C = KD/L^2$$

where a = rate of O_2 consumption per unit volume,
C = O_2 concentration at surface of the tissue,
D = diffusion coefficient of O_2 in the tissue,
L = distance from centre to surface of the tissue,
K = a constant depending only on the shape of the tissue.

An anoxic core develops in the tissue whenever a/C is increased above the critical value defined by the above equation. The present significance of the equation is that for a given tissue of any fixed size, shape, and oxygen diffusion coefficient, anoxia appears when a/C is increased to the same critical value whether this is done by raising a (as with activity) or by lowering C (as when replacing

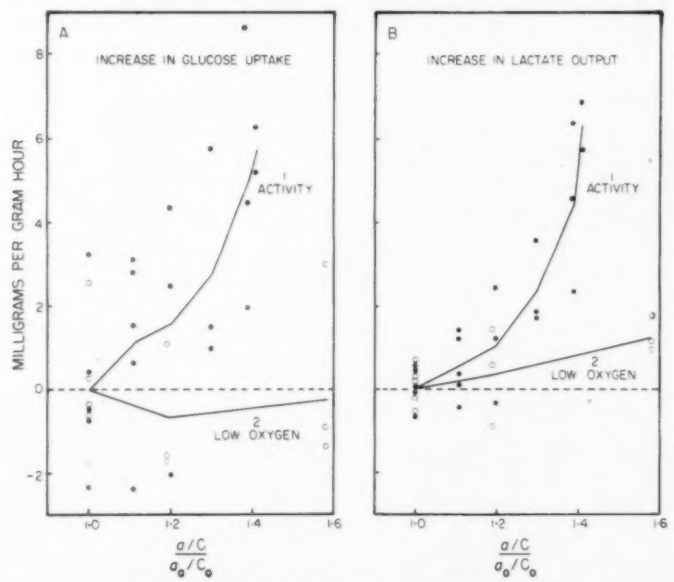

FIG. 6.—Changes in glucose uptake and lactate output associated with increased rate of oxygen consumption during activity (filled circles) and with reduced concentration of oxygen in the bathing fluid (unfilled circles). *Abscissa:* a = rate of oxygen uptake during activity, and a_0 = rate at rest, both from data of LARRABEE (1957). C = percentage of oxygen in the equilibrating gas. C_0 = control percentage of oxygen (= 95 per cent). For the upper curves $C = C_0$. For the lower curves $a = a_0$ (neurons at rest). For points at abscissa = 1·0, the preparation was at rest with 95% O_2. All changes in rates for glucose and lactate are corrected for spontaneous changes with time. Lines are drawn through average values.

oxygen with nitrogen). Therefore effects of activity can be appropriately compared with those of reduced oxygen concentration by plotting against an abscissa which is proportional to a/C. This has been done in Fig. 6. In constructing the graphs concerning activity, the rate of oxygen uptake was obtained by interpolation of previously published data (LARRABEE 1957) for each frequency at which glucose metabolism was measured. This permitted calculation of the value for the abscissa.

It is apparent that the rates for both glucose and lactate were changed insignificantly by lowering the oxygen concentration, compared to the changes accompanying similar alterations in a/C during activity. This indicates that the increased metabolic rates during activity cannot be ascribed to anoxia.

Diffusion of oxygen has thus been shown to be adequate during activity as well as during rest. Compared to oxygen the external concentration of glucose was higher (5·5 compared to about 1 mM) and the rate of utilization of glucose was lower (65 compared to 300 μmoles/g/hr). These large differences in concentration and utilization are more than enough to compensate for the somewhat slower diffusion

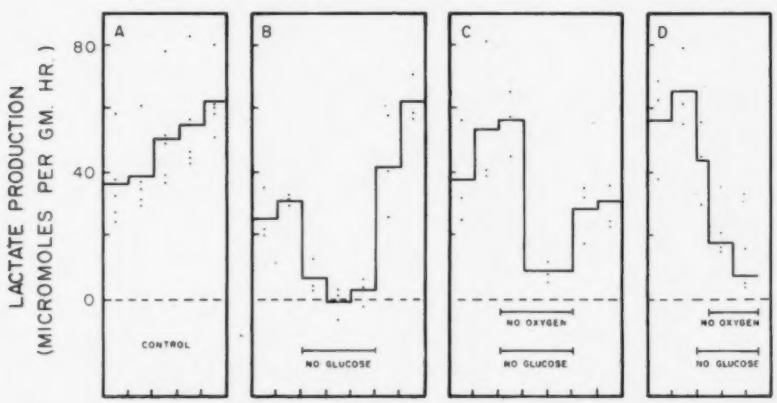

FIG. 7.—Changes in rate of lactate production caused by withdrawal of glucose and by withdrawal of both glucose and oxygen. Points represent individual determinations. Lines are drawn through averages. Markers at bottom indicate time in hours.

of glucose due to its larger molecular weight, since the measured diffusion coefficient for oxygen in aqueous solution is only about 3 times that for glucose (HÖBER, 1945). Therefore it may be assumed that the diffusion of glucose was also sufficient.

Evidence concerning sources of lactate. It is generally accepted that lactate is formed metabolically by the reduction of pyruvate. Therefore questions concerning the origin of the lactate which appeared in the bathing fluid are best considered in terms of possible origins of pyruvate. Moreover the rate at which lactate is formed will obviously depend not only on the rate of formation of pyruvate, but also on the rate at which pyruvate is converted into materials other than lactate.

Under the conditions of our experiments pyruvate could have been derived either from the glucose taken from the bathing fluid or from various substrates endogenous to the preparation. The following experiments indicate that glucose was the major source, at least in resting ganglia.

(1) When *glucose* was withdrawn by exchanging the usual bathing fluid for one from which glucose had been omitted, the rate of lactate production fell rapidly and was soon below the limits of detection by our methods (Fig. 7B). This showed that glucose was an important source of lactate. However, it did not prove that glucose was the only source, since it is at least possible that lactate appears only when the rate of pyruvate formation, from all sources combined, exceeds the minimum rate at which pyruvate can be oxidized. This limiting rate might possibly be reached only in the presence of an external supply of glucose. Therefore we have further

investigated the problem by stopping the oxidative removal of pyruvate by withdrawal of oxygen.

(2) When *oxygen* was removed by equilibrating the solution with 95% N_2, 5% CO_2, with glucose still present, rates of both glucose uptake and lactate production increased several-fold above the normal levels. Moreover the two rates became equal to one another within the rather good limits of accuracy of these particular experiments (Table 4, last line). This is in agreement with a strictly glycolytic source of lactate, since by stopping oxidative removal of pyruvate, most of that which is formed would be expected to appear as lactate. Thus at least under conditions of anoxia, glucose is the only important source of material for formation of lactate.

(3) When *glucose and oxygen* were simultaneously withdrawn, lactate production fell to a low rate, although not during the first hour (Fig. 7C). Calculations suggested that the delay might be due to production of lactate from glucose which had previously entered the tissue. In agreement with this conclusion was the observation that lactate production fell more rapidly after oxygen withdrawal if glucose had been removed half-an-hour earlier (Fig. 7D). In these latter experiments the ganglion was immersed for ten minutes in each of three successive batches of glucose-free solution, which were pooled for subsequent chemical analysis. Oxygen was then also withdrawn and the usual one-hour sampling procedures were resumed.

After performing a variety of experiments, it may thus be concluded that no evidence has yet been found to suggest that any important fraction of the lactate appearing in the bathing fluid is derived from a source other than the glucose taken up. It will therefore be assumed in the Discussion that the lactate comes entirely from the glucose which has been consumed.

DISCUSSION

Metabolism at rest. Our results on resting ganglia are in agreement with the assumptions that all of the lactate appearing in the bathing fluid was formed from the glucose which was consumed, and that the remainder of the glucose was all completely oxidized to CO_2 and water.

Evidence favouring the assumption that the lactate came from glucose has already been presented in the last section on Results. The conclusion that the remainder of the glucose was oxidized is supported by calculations presented in Table 5, which show that the amount of oxygen which would have been required for this purpose was approximately equal to the amount which was actually consumed. The required rate of oxygen uptake was calculated from the stoichiometric relationship that six molecules of oxygen are required for complete oxidation of one molecule of glucose. The rate of oxygen uptake thus calculated differed by only 9 per cent from the actual rate, which was measured on similar ganglia and reported elsewhere (LARRABEE, 1957). This is considered to be a good agreement, in view of the small amounts of tissues employed in these studies and the fact that there were some systematic differences between the conditions for measurement of glucose metabolism and for measurement of oxidation. Moreover the difference was not statistically significant. It is thus reasonable to conclude that glucose is the major substrate for oxidation when the neurons are at rest in a sympathetic ganglion. This is in agreement with the conclusions of other investigators for central nervous tissues and for peripheral nerve trunks.

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Metabolism during activity. It has previously been reported that the rate at which oxygen is consumed by a sympathetic ganglion increases when the neurons are made active by electrical stimulation of the preganglionic nerve (LARRABEE and BRONK, 1952; LARRABEE, 1957). In the present experiments we have shown that the rate of

TABLE 5.—METABOLIC BALANCE AT REST IN GANGLION PREPARATIONS (INCLUDING NERVES)

Mean ± S.E.M. in μmoles/g/hr (dry wt.).
Number of preparations in parentheses.

Glucose remainder (= glucose uptake minus lactate output)*	45·7 ± 2·3 (46)
Oxygen required for complete oxidation of glucose remainder	274 ± 14 (46)
Oxygen actually consumed (LARRABEE, 1957)	300 ± 9 (23)
Oxygen consumed in excess of calculated requirement	26
t-test of oxygen excess	$0{\cdot}3 > P > 0{\cdot}2$ (Not significant)

* From last line in Table 1, converted to molar units.

glucose uptake is also accelerated during activity. However it appears that the extra glucose does not provide substrate for the additional oxygen, since the rate of lactate output increased as much as did the rate of glucose uptake. Thus intracellular materials must undergo oxidation in an active ganglion. The rate of this oxidation is indicated by the extent to which the rate of oxygen uptake exceeded the rate accounted for by glucose metabolism. This is shown by the separation between the curves in Fig. 8. It is apparent that the rate of oxidation of intracellular substrates must have increased progressively with the frequency of stimulation.

In order to obtain a statistical test of the difference between the oxygen uptake observed during stimulation and that calculated from the glucose and lactate data, a comparison was made of (a) the averages of oxygen uptakes measured at both 8 and 15 per sec in each of 5 experiments (406 ± 36 μmoles/g/hr, mean ± S.E.M., $n = 5$) and (b) the uptakes calculated from glucose and lactate measurements at some one frequency between 5 and 15 per sec in each of eight experiments (271 ± 26 μmoles/g/hr, mean ± S.E.M., $n = 8$). According to a *t*-test, the difference (135 μmoles/g/hr) was significant at the 2 per cent level. When the increments caused by stimulation, rather than the absolute rates, were similarly compared, the difference was found to be significant at the 1 per cent level. These statistical tests thus confirm the impression conveyed by Fig. 8 that oxygen was indeed consumed in excess of that required for oxidation of the available glucose.

Our results thus agree with evidence obtained by HOLMES *et al.* (1930), as well as by MULLINS (1953), in studies on frog and rabbit nerves, indicating that glucose is not the substrate for the activity increment in oxygen consumption in excised nervous tissue. We have extended their conclusion to a tissue containing not only nerve

fibres but also nerve cell bodies and synaptic contacts, and also to a species not investigated by the previous workers. In contrast with the older studies we find that glucose uptake is accelerated during activity. However, the final conclusion concerning the activity substrate is the same, since substances other than glucose appear to be oxidized.

GEIGER, MAGNES and DOBKIN (1954) have reported observations in perfused cat brain which appear to be related to ours. They found that 'carbohydrate breakdown in the brain is accelerated during convulsions, but all loss is accounted for as lactic acid, despite the usual increase of oxygen consumption.' A suggestion of similar relationships in excised brain tissue exposed to electrical pulses is contained in a single experiment reported by MCILWAIN, ANGUIANO and CHESHIRE (1951).

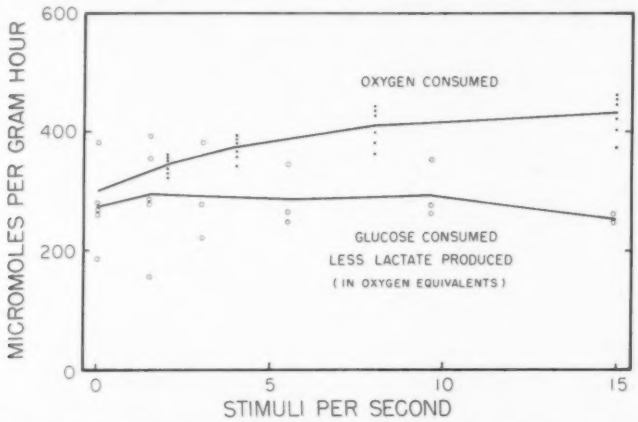

FIG. 8.—Comparison of rate of oxygen consumption with the calculated rate of glucose oxidation at various frequencies of neuronal activity. Lines are drawn through averages. *Lower lines and unfilled points:* Rate of glucose oxidation, calculated by subtracting the rate of lactate output from the rate of glucose uptake. The remainder was assumed to be completely oxidized to CO_2 and H_2O. *Upper lines and filled points:* Rate of oxygen consumption, as previously reported (LARRABEE, 1957). The mean resting rate at 36°, measured in 23 experiments, was multiplied by the percentage increments found during stimulation of the preganglionic nerve at several frequencies in each of 6 experiments at 36°.

Thus the failure of glucose metabolism to account for oxygen uptake during activity in ganglia is shared by other tissues, at least under certain experimental conditions.

The nature of the endogenous substrate oxidized during activity. As stated above, MULLINS found no acceleration in the rate of CO_2 production from glucose labelled with ^{14}C during activity in frog nerve. However, the same investigator observed an increase in production of radioactive CO_2 during activity in nerves which had been soaked in labelled glycine, alanine or glutamate. Since this suggests that certain amino acids may be oxidized in preference to glucose during activity, evidence for amino acid utilization was sought in rat ganglia by measuring the output of ammonia (STEKIEL, 1957; STEKIEL and LARRABEE, 1957; LARRABEE *et al.*, 1957). Ammonia was indeed produced at a measurable rate, even in resting ganglia, the mean $\pm$ S.E.M. in sixteen resting preparations being $2{\cdot}2 \pm 0{\cdot}5$ μmoles/g/hr (dry weight). However, there was no detectable increase in the rate of ammonia production during activity, whereas a rather large increase might have been expected. If, for example, in attempting to account for an excess oxygen uptake amounting to 120

μmoles/g/hr during activity, we assume complete oxidation of some one of the amino acids implicated by MULLINS, then at least 27 μmoles of nitrogen should be released per g/hr. This would be more than 12 times the resting rate and therefore would have been detected easily if it had appeared as free ammonia. Unfortunately the possibility must be considered that this much amino nitrogen is indeed involved, but is retained chemically within the tissue by one or more of several well-known mechanisms, instead of being released as ammonia. These ammonia measurements must therefore be interpreted as an unsuccessful attempt to demonstrate an increased breakdown of amino acids during activity rather than as conclusive evidence against such increased breakdown. Thus the endogenous substrate oxidized during activity remains unidentified.

Speculations concerning metabolism in situ. The conclusion reached in this paper, that materials endogenous to a ganglion are burned during activity, can not be extended without qualification to a ganglion *in situ*. Certainly such a tissue is normally dependent on materials reaching it through the blood and must utilize these either directly or after conversion into other chemical compounds. This raises a question concerning the implications of our findings.

One possibility is that substances other than glucose are normally delivered by the blood, stored in the tissue, and oxidized during activity *in situ* as well as after excision. Alternatively oxidations *in situ* may be restricted almost exclusively to glucose, other materials being oxidized during activity only after excision. Such a shift in substrate utilization might be caused, for example, by omission from the bathing fluid of some factor needed for sufficiently rapid oxidation of pyruvate.

The suggestion that only carbohydrate may be oxidized under natural conditions receives some indirect support from the recent discovery of experimental circumstances under which the activity increment in ganglionic oxygen consumption could indeed be accounted for by carbohydrate metabolism (DOLIVO and LARRABEE, 1957*a*, *b*, *c*). This was accomplished by reducing both temperature and pH. The suggestion that oxidations are normally limited to carbohydrate may also explain the significance of the accelerated uptake of glucose: this might normally serve to provide additional substrate for oxidative generation of energy, even though this additional oxidation may be blocked under certain experimental conditions.

These speculations indicate that it is not impossible to reconcile our results with the contention of some investigators that oxidation in nervous tissue is normally concerned almost exclusively with glucose as substrate. Nevertheless it should be emphasized that intracellular materials were certainly oxidized during activity under the carefully controlled conditions of the present experiments. The possibility of non-carbohydrate oxidation by active neurons *in situ* therefore deserves further consideration.

SUMMARY

Rates of glucose uptake and lactate output were measured at 36° in superior cervical sympathetic ganglia excised from rats. This was done in resting preparations and also when the ganglionic neurons were in action at various frequencies as a result of electrical stimulation of the preganglionic nerve. Action potentials in the postganglionic nerve were measured throughout each experiment. Preganglionic stimulation was always supramaximal.

The rate of glucose uptake increased progressively with the frequency of neuronal

activity. The increase amounted to as much as 40 per cent of the resting rate at a frequency of 15 per sec. The rate of lactate production increased by about the same absolute amount as did the rate of glucose uptake at all frequencies. This is interpreted to imply that all the extra glucose taken up during activity was converted to lactate, so that no more glucose was available for oxidation during activity than during rest.

Control experiments indicated that the results were not significantly influenced by limitation of oxygen diffusion into the tissue. There was also evidence to support the assumption that the lactate put out by the tissue was formed from the glucose which was taken up.

It is concluded that the resting rate of oxygen uptake previously reported for the superior cervical ganglion of the rat can be quantitatively accounted for by assuming that oxygen is used only for burning the glucose taken from the bathing fluid, due allowance having been made for the amount of glucose converted to lactate. During activity, however, some substrate endogenous to the excised tissue must be oxidized, since a considerable increase in rate of oxygen uptake is caused by stimulation, whereas the extra glucose which is concurrently consumed is apparently all converted to lactate.

The possible nature of the endogenous substrate oxidized during activity, and the possible relationships of these findings to ganglionic metabolism *in situ* are considered in the Discussion.

APPENDIX

Methods of chemical analysis

(*a*) *Glucose analysis.* Duplicate aliquots of experimental and control bathing fluids and triplicates of standards known to contain 0, 6 and 10 μg of glucose were analysed by the method of PARK and JOHNSON (1949) with minor modifications. This method depends on the reduction of ferricyanide to ferrocyanide by the glucose, and subsequent formation of Prussian blue (ferric-ferrocyanide) after addition of ferric ions. The Prussian blue was measured quantitatively in a Beckman Model B spectrophotometer at a wavelength of 690 mμ. The following minor modifications were made in the procedures described by PARK and JOHNSON in order to improve reproducibility. (1) The samples were kept in the dark during as much of the time as possible. (2) The knowns and unknowns were analysed in a sequence which, by the use of arithmetical means, eliminated the effects of a drift in optical density which was found to progress during the spectrophotometric readings. (3) The concentration of Duponol was increased to 1½ times the level recommended by PARK and JOHNSON.

The sensitivity, in micrograms of glucose per optical density unit, was calculated from the results on the known samples. The amount of glucose removed by the tissue could then be calculated from the difference between the optical densities developed by bathing fluid which had been exposed to the tissue and by control samples of the same fluid which had not been thus exposed.

Analysis of the known samples showed that the density was a linear function of the glucose concentration, and that it was unaffected by adding lactate in an amount more than 30 times that ordinarily contributed by the tissue.

As in all experiments where reducing methods are employed for glucose analysis, the possibility of interference by other reducing substances must also be considered. Two lines of evidence, presented in another context in this paper indicate that interference of this sort did not occur to a significant extent: (1) There was no significant difference between rates of glucose uptake reported here and those found earlier using a method based on glucose oxidase, which is highly specific for glucose (EDWARDS and LARRABEE, 1955). (2) In the absence of oxygen, the rate of glucose uptake, as measured by the reducing methods employed in this paper, became equal to the rate of lactate production, in agreement with predictions of contemporary biochemistry.

(*b*) *Lactate analysis.* Single aliquots of the fluids exposed to the tissue and of samples of bathing

fluid which had not been thus exposed, as well as samples in duplicate known to contain 0, 1, 2 and 3 μg of lactate (as zinc lactate), were analysed by a modification of the method of BARKER and SUMMERSON (1941). This depends on conversion of lactic acid to acetaldehyde by heating in concentrated sulphuric acid and the later development of colour by treatment with *p*-hydroxydiphenyl in the presence of cupric ions in the concentrated acid. Optical density, determined at 565 mμ, varied linearly with amount of lactate.

The major modifications in the method of BARKER and SUMMERSON were the omission of deproteinization, and omission of the further removal of interfering materials by precipitation with copper sulphate and calcium hydroxide. These steps were eliminated in order to get higher yields in the development of colour and thus to increase the sensitivity of the method. It was also hoped to improve reproducibility under our conditions through this simplification. Justification for omitting removal of protein has been offered in the Methods section. Other interfering substances should be present only in insignificant quantities in the small volumes of fluids which had been exposed to such small amounts of tissue. Moreover, MILLER and MUNTZ (1938) showed that the method had considerable specifity even when the precipitation in copper sulphate and calcium hydroxide was omitted.

Addition of glucose, in an amount approximately equal to that normally contributed by the bathing fluid, increased the optical density by an amount equivalent to only about 0·03 μg of lactate. Therefore the typical 10 per cent difference between the glucose contents of control fluid and fluid exposed to the tissue could cause an error in determination of lactate in an aliquot by only about 0·003 μg. This was negligible since an aliquot ordinarily contained at least 100 times this much lactate. Addition of 5 μg of pyruvate caused no interference; this amount was more than 5 times that which could appear in the samples even if all the glucose consumed were converted into this substance alone.

The sensitivity, in micrograms of lactate per optical density unit, was determined from results on the samples of known content. The amount of lactate produced by the ganglion was then calculated from the difference in optical densities generated by control bathing fluid and by fluid which had been in contact with the tissue.

(*c*) *Other details of chemical methods.* All chemical steps and spectrophotometric measurements were made in Pyrex culture tubes, 125 × 15 mm, which had been calibrated and selected for optical path lengths matching to within 0·7 per cent.

A water cell with plate glass windows was placed in the optical path of the spectrophotometer, so that absorption could be measured while the tubes were immersed in water. This minimized effects of irregularities in their surfaces. The water cell also provided the additional advantage that only a small deflection was caused by insertion of a water-filled test tube, so that the zero for absorption could be reset before each reading without the usual necessity of repeatedly inserting a control tube.

In order to prevent loss of water by evaporation during steps in which test tubes were placed in a boiling water bath, the tops were closed with condensers made from medicine droppers having glass bulbs in the stems. The bottoms of the stems, which hung down into the test tubes, were sealed, and the droppers were filled with water. The steam of the water bath was prevented from condensing and entering the test tubes by the use of a baffle and by blowing the steam away with a fan.

(*d*) *Precision.* By pooling the differences between duplicate analyses throughout the course of the experiments, and by following the method of calculation described by YOUDEN (1951), the standard deviation of a determination on an aliquot was found to be 0·11 μg for glucose and 0·05 μg for lactate. This corresponded to 0·40 μg of glucose and 0·18 μg of lactate in the total sample collected from the tissue. Because each calculation of rate of glucose uptake depended on the difference between the mean of 4 analyses of control bathing solution and 2 analyses of solution which had been exposed to the tissue, it could be calculated that the standard deviation of the result was about 0·4 μg of glucose, or about 10 per cent of the glucose consumed by a typical ganglion in one hour at rest. Similarly the standard deviation of an experimental determination of rate of lactate production was calculated to be about 0·2 μg of lactate, or about 20 per cent of the lactate typically produced per hour at rest.

Acknowledgement—It is a pleasure to acknowledge the technical assistance of Miss EDITH KOLACSKOVSZKY, who carefully made the glucose and lactate analyses, almost *ad infinitum*.

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THE CHOLINE ACETYLASE ACTIVITY OF RABBIT BRAIN

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(*Received* 27 *July* 1957)

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THE synthesis of acetylcholine (ACh) is now known to proceed by two steps: in the first step, acetyl coenzyme A (acetyl CoA) is formed and contributes acetyl groups for the second step, the acetylation of choline, catalysed by choline acetylase (KORKES, DEL CAMPILLO, KOREY, STERN, NACHMANSOHN and OCHOA, 1952). The activity of choline acetylase, as measured by the rate of synthesis of ACh, is therefore dependent on the presence and activity of the system forming acetyl CoA. SMALLMAN (1956) showed that the rate of formation of acetyl CoA was, in fact, limiting on the rate of synthesis of ACh in a preparation from blowfly heads; the initial rate of synthesis was about ten-fold greater when pre-formed acetyl CoA was present than when acetyl CoA was formed coincidently with the acetylation of choline. Since the latter method has been used in most studies on the rate of ACh synthesis by mammalian brain, it seems pertinent to enquire whether the rates so obtained do in fact reflect the activity of the terminal enzyme, choline acetylase. In the present study, information about the activity of choline acetylase from rabbit brain has been obtained by using a variety of methods to supply the enzyme with acetyl CoA.

METHODS

Rabbits were killed by a blow on the neck, the brains excised immediately, and the parts rostral to the superior colliculi used for the preparation of acetone powders. The tissue was ground in a cold mortar in acetone at $-4°$ and suspended in a volume of acetone 90 to 100 times the volume of tissue. The acetone suspension was filtered in the cold, and the resultant powder was dried over P_2O_5 in an evacuated desiccator at 4°. The powders were extracted in cysteine saline solution (2·5 mg L-cysteine hydrochloride per ml, 0·9 per cent NaCl, neutralized with 0·33 N-NaOH) at concentrations of 50–100 mg powder per ml, and were used immediately or kept frozen for 2 or 3 days before use. Extracts were centrifuged at 3,000 rev/min for 5 min before use.

Extracts were incubated in small flasks shaken in a Warburg respirometer bath at 37°. Unless otherwise stated, the final concentrations in the incubation mixtures were as follows (μmoles/ml): L-cysteine, 20; $MgCl_2$, 5; KCl, 27; eserine sulphate, 0·25; phosphate buffer of pH 6·8, 7; sodium acetate, 10; adenosine triphosphate (ATP), 1·5; Pabst CoA, 0·1; choline, 10; and extract equivalent to 5 mg acetone powder/ml. The total volume was usually 10 ml and the rate of ACh synthesis was determined by withdrawing 2 ml aliquots at the specified intervals after addition of choline to the otherwise complete incubation mixture. An aliquot was withdrawn immediately and used to correct all subsequent values for ACh present at zero time. All aliquots were immediately pipetted into 0·5 ml of 0·33 N-HCl to stop the reaction.

The ACh content of incubates was assayed on the eserinized frog's rectus abdominis muscle, correcting for the presence of sensitizing substances (FELDBERG, 1950). All values refer to ACh chloride.

The pigeon liver enzyme extract used in this study was supplied by Dr. C. O. HEBB and prepared as already described (HEBB, 1955). Acetyl CoA was prepared by acetylation of Pabst CoA with acetic anhydride, purified by paper chromatography and measured spectrophotometrically, as detailed by NOVELLI, in the compilation by GLICK (1955).

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In one experiment, acetyl CoA produced by a preparation of blowfly heads was used to provide acetyl CoA for rabbit brain extract. This was done by incubating an extract of acetone powder of blowfly heads with CoA, ATP, acetate, and all other components of the incubation mixture, except choline, for 20 min and then acidifying to pH 2–3 for 30 min to inactivate the enzymes. This preparation was then neutralized and used as the source of acetyl CoA with brain extracts provided with choline as the only other addition to the incubate. To verify this method of providing acetyl CoA it was necessary to show that blowfly extracts supplied only with this preparation and choline produced ACh at about the same rate as blowfly extracts supplied with all components (CoA, ATP, acetate and salts) and incubated in the usual way without interruption by the acidification step. Accordingly, an extract of blowfly heads was incubated with all components except choline for 20 min and then divided into two aliquots. Choline was added immediately to one aliquot, which then produced 5·1 mg/ACh/g acetone powder during the next 15 min. The other aliquot was acidified, neutralized and incubated in equivalent concentrations with active blowfly extract and choline. This incubate yielded 4·8 mg ACh/g/15 min indicating that the inactivated aliquot contained acetyl CoA approximately equivalent to the amount present at the end of the 20 min induction period.

RESULTS

The rate-limiting effect of acetyl coenzyme A formation

The first step was to establish whether the formation of acetyl CoA by rabbit brain extracts was rate-limiting on the activity of choline acetylase in the extracts. The method developed previously (SMALLMAN, 1956) was used. Extracts of rabbit

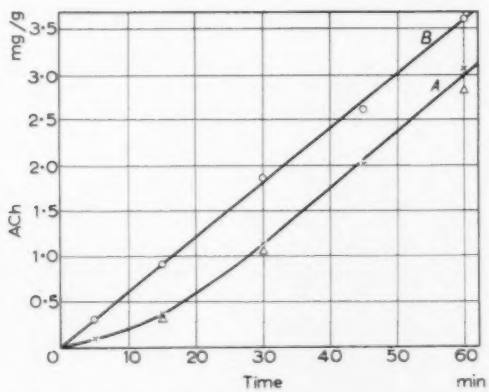

FIG. 1.—The rate of synthesis of ACh by brain extract. Curve A, all components present at zero time; curve B, all components except choline present during a 20 min induction period, choline added at zero time; triangles plotted on curve A, extract incubated for 20 min before adding all other components at zero time.

brain were provided with the components required for acetyl CoA formation (CoA, acetate, ATP) during an induction period without choline. Subsequently, choline was added and samples withdrawn at intervals to determine the rate of ACh synthesis. For comparison, an aliquot of the same extract was set up in the same way except that choline was provided simultaneously with the components required for acetyl CoA formation. Thus, if the rate of acetyl CoA formation was limiting on the rate of ACh synthesis, ACh should be formed at a slower rate when all components were added together than when acetyl CoA was allowed to form and accumulate during an induction period before the addition of choline.

Fig. 1 shows that the rate of acetyl CoA formation by rabbit brain extracts is, in

fact, limiting on the rate of ACh synthesis. When acetyl CoA is allowed to accumulate during an induction period (curve B), the initial rate of ACh synthesis was 2–3 times higher than it is when the acetylation of choline is dependent on the coincident formation of acetyl CoA (curve A). In the second case, however, the figure shows that the rate of ACh synthesis gradually increases until between 20 and 30 min it becomes identical with that obtained after an induction period for the formation of acetyl CoA. This initial lag has been observed by others and attributed to the time required for the accumulation of some substance essential for the synthesis of ACh (KOREY, DE BRAGANZA and NACHMANSOHN, 1951). It may now be presumed that this substance is acetyl CoA, and that during the initial stages of the reaction its concentration is too low to allow its full utilization for ACh production by choline acetylase; subsequently, acetyl CoA reaches a critical concentration at which ACh

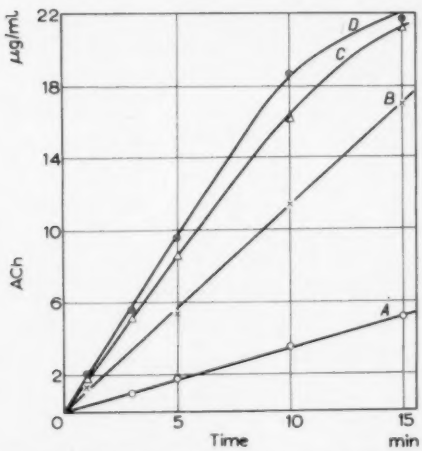

FIG. 2.—The effect of extract concentration on rate of ACh synthesis. Curve A, extract of 5 mg acetone powder/ml incubate; B, 15 mg/ml; C, 25 mg/ml; D, 35 mg/ml. Induction period for 20 min before addition of choline at zero time.

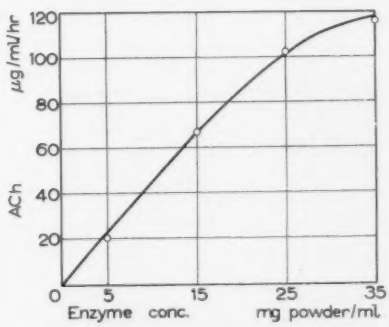

FIG. 3.—The relation between extract concentration and choline acetylase activity. Data derived from the initial rates of ACh synthesis shown in Fig. 2.

is synthesized at a linear rate. When conditions are provided for the formation of acetyl CoA prior to the addition of choline (curve B) the lag is eliminated and ACh is synthesized at a linear rate throughout the observed period.

Fig. 1 also shows that the activity of the ACh synthesizing system was unimpaired during the induction period. When the extract was incubated for 20 min prior to the addition of all other components at zero time (triangles plotted on curve A), the results were the same as when the extract was introduced at zero time, without pre-incubation.

The relation between extract concentration and choline acetylase activity

Widely different concentrations of brain extracts have been used in studies of ACh synthesis, and it seemed important therefore to establish the relation between extract concentration and choline acetylase activity.

The results are shown in Figs. 2 and 3. An induction period for the formation

of acetyl CoA was used throughout, so that the initial rates of ACh synthesis express the activity of choline acetylase. Fig. 2 shows that acetone powders of rabbit brain at concentrations of 5 and 15 mg/ml synthesized ACh at a linear rate during 15 min after the addition of choline. At higher concentrations, however, the rate of ACh formation slowed sharply after 10 min, presumably because the supply of pre-formed acetyl CoA has been exhausted. When the initial rates obtained in these experiments are plotted against extract concentration (Fig. 3), a linear relation up to a concentration of 25 mg/ml is revealed; at higher concentrations the rate of ACh formation does not increase proportionately.

These results show that the activity of choline acetylase is directly proportional to its concentration over a considerable range of extract concentrations. Within this range, estimates of the activity of choline acetylase, determined at different extract concentrations, will be equally valid. At very high concentrations, however, it appears that the activity of the enzyme may be underestimated. The activity may be underestimated also unless care is taken to establish the initial rate of synthesis. For instance, it is clear from Fig. 2, curves C and D, that the values obtained at 15 min would indicate activities considerably less than those established by the initial rates. Both sources of error have been avoided in this study by using extracts at a concentration of 5 mg acetone powder/ml.

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Effects of various methods of supplying acetyl CoA

From Figs. 1, 2 and 3 the activity of the choline acetylase of rabbit brain is estimated as yielding 3·6–4·0 mg ACh/g acetone powder per hr. These values were obtained by allowing the brain extract itself to form acetyl CoA during an induction period. Other methods for supplying acetyl CoA were now used to determine whether these rates are in fact optimal.

Acetyl CoA may be formed by the acetate-activating enzyme using acetate as substrate (JONES, BLACK, FLYNN and LIPMANN, 1953), or by the condensing enzyme using citrate as substrate (STERN, SHAPIRO, STADTMAN and OCHOA, 1951), and both enzymes appear to be present in brain extracts (BALFOUR and HEBB, 1952). Moreover, when both these systems were invoked by providing acetate and citrate together, the rate of ACh synthesis was the sum of the rates with each separately, using a preparation from blowflies with extremely high choline acetylase activity (SMALLMAN, 1956). This method for supplementing acetyl CoA formation was therefore applied in the present study. A second method was to supplement the brain extract with acetyl CoA produced during an induction period by the highly active blowfly preparation. A third method involved supplementing the brain extract with pigeon liver extract for the formation of acetyl CoA (HEBB, 1955). Finally, brain extracts were supplied with acetyl CoA produced by the chemical method detailed by GLICK (1955).

The results presented in Table 1 show that whatever method was used to provide acetyl CoA, the optimal rate of synthesis of ACh by the choline acetylase of brain extract was about 4 mg/g acetone powder per hr. With acetate as acetyl donor (line 1), the acetate-activating enzyme of brain extract appears capable of producing, during the induction period, sufficient acetyl CoA to saturate the choline acetylase, for ACh was synthesized at the optimal rate. Although synthesis also occurred with citrate (line 2), the value obtained with citrate and acetate together was not significantly higher than with acetate alone. When the brain extract was supplemented with the

acetyl CoA producing enzymes of aged pigeon liver extract, the value was no higher (line 4). Acetyl CoA produced during an induction period with an extract of blowfly heads did not increase the rate of synthesis by the brain extract although, when added to blowfly extract itself, it supported a rate of synthesis five times greater (line 5).

TABLE 1.—EFFECT OF VARIOUS METHODS FOR FORMATION OF ACETYL CoA ON SYNTHESIS OF ACETYLCHOLINE BY RABBIT BRAIN EXTRACT

Method for formation of acetyl CoA	ACh mg/g powder/hr
(1) Induction period*, brain extract + acetate	4·2
(2) Induction period, brain extract + citrate	1·8
(3) Induction period, brain extract + acetate + citrate	4·3
(4) Induction period, pigeon liver extract + acetate + citrate	4·0
(5) Blowfly extract, inactivated after induction period†	3·8
(Same preparation incubated with active blowfly extract)	19·2
(6) Chemically produced acetyl CoA, 0·2 μmoles/ml	1·8

* Incubation of extract with ATP, CoA and acetate or citrate for 20 min before addition of choline.
† See Methods.

Finally, chemically-produced acetyl CoA at twice the concentration theoretically possible with the enzymic methods (CoA, 0·1 μmoles/ml) was only about half as effective as when acetyl CoA was provided by the enzyme systems.

The activity of choline acetylase with chemically-produced acetyl CoA

From these results, the optimal rate of synthesis of ACh was about 4 mg/g/hr when acetyl CoA was provided by enzymic methods; but when chemically-produced acetyl CoA was used, the rate of ACh synthesis was reduced by half. This anomaly had been noted previously by BERMAN, WILSON and NACHMANSOHN (1953), and was investigated further in this study.

Fig. 4 confirms the discrepancy indicated in Table 1. The rate of ACh formation with the enzymic production of acetyl CoA was nearly twice that obtained with chemically-produced acetyl CoA, even though in the latter case the provision of 0·1 μmoles/ml acetyl CoA equals the maximum obtainable with the enzymic system provided with 0·1 μmoles/ml CoA. The lower rate with chemically-produced acetyl CoA might result from the absence of ATP from this system and the consequent inability to re-acetylate CoA following the transfer of acetyl groups from acetyl CoA to choline. The figure shows however that the addition of ATP made no difference to the rate of ACh synthesis.

Finally, the activity of choline acetylase at various concentrations of chemically-produced acetyl CoA was measured to determine whether this relation conformed with conventional enzyme-substrate kinetics, and to enable calculation of the maximal activity of choline acetylase.

Fig. 5a shows the initial rates of ACh synthesis obtained at various concentrations of acetyl CoA. Under these conditions, the choline acetylase of rabbit brain was nearly saturated with respect to acetyl CoA at a concentration of 0·2 μmoles/ml.

In Fig. 5b these data are plotted according to the method of LINEWEAVER and BURK (1934). In conformity with normal enzyme-substrate relation, the data yield a straight line with slope indicating a Michaelis-Menten constant of $2{\cdot}2 \times 10^{-5}$ M. From the intercept of the line, the maximal velocity of the reaction catalysed by

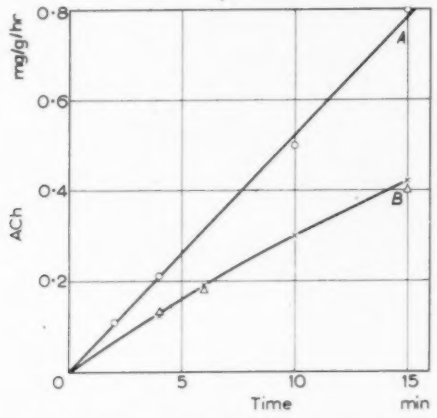

FIG. 4.—The rate of ACh synthesis with chemically and enzymically-produced acetyl CoA. Curve A, induction period with ATP, acetate, salts and CoA (0·1 μmoles/ml), choline added at zero time; curve B, incubation with salts, choline and acetyl CoA (0·1 μmoles/ml); triangles plotted on curve B, same conditions as before but with ATP present.

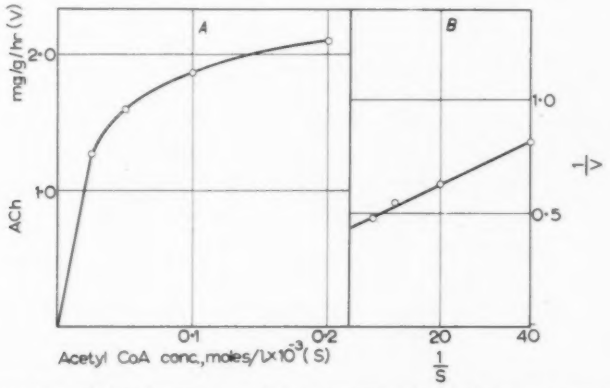

FIG. 5.—(*a*) The relation between acetyl CoA concentration and choline acetylase activity. Points represent the initial rates of ACh synthesis obtained during incubation of extract plus salts with only choline, and chemically-produced acetyl CoA at the indicated concentrations. The initial rates were estimated from curves, each with four values obtained at about 4 min intervals during the first 15 min of the reaction.
(*b*) Derivation of the Michaelis-Menten constant, $2{\cdot}2 \times 10^{-5}$ M, for enzyme with respect to acetyl CoA.

choline acetylase may be calculated to yield 2·4 mg ACh/g/hr. The highest observed value was 2·1 mg ACh/g/hr (Fig. 5*a*) and the curve indicates that slightly higher values would be expected with higher concentrations of acetyl CoA. These results confirm therefore that, when chemically-produced acetyl CoA is supplied at optimum concentration, the rate of ACh synthesis by brain extracts is little more than half the rate obtained with enzymically-produced acetyl CoA.

DISCUSSION

This study was undertaken to test the possibility that previous studies had underestimated the activity of the choline acetylase of brain extracts. This possibility became evident from the study of a highly-active preparation of blowfly heads in which it was shown that the rate of formation of acetyl CoA by extracts was limiting on the activity of choline acetylase, and furthermore that the activity was grossly underestimated unless care was taken to determine the initial rates of ACh synthesis (SMALLMAN, 1956).

It has now been shown that with brain extracts also, the activity of choline acetylase was limited by the rate of acetyl CoA formation by the extracts. Without an induction period for the formation of acetyl CoA, there was an initial lag in the rate of ACh synthesis, and the value obtained after a 1 hr incubation was 15 per cent less than the rate found when acetyl CoA was provided during an induction period. Even with an induction period, the activity of choline acetylase may be underestimated unless the initial rates of ACh formation are determined. This was shown in the study of the effect of extract concentration on activity. At high extract concentrations, the initial linear rate of ACh synthesis was sustained for 10 min and then declined sharply. Similar results were obtained in studies with a highly-active preparation of blowfly heads in which the high initial rate of ACh formation was maintained for only 2–3 min (SMALLMAN, 1956).

The activity of the choline acetylase of rabbit brain extracts was estimated at about 4 mg/ACh/g acetone powder per hr when acetyl CoA was provided by the induction method. This rate appears to be optimal, for when acetyl CoA was provided by the several other enzymic methods used, no higher values were obtained. Especially convincing was the finding that a preparation of acetyl CoA formed by blowfly extracts failed to increase the rate of synthesis by brain extract, although it contained sufficient acetyl CoA to support a rate five times greater when added to blowfly extract itself. These findings lead to the conclusion that the acetyl CoA forming system in extracts of rabbit brain is able to produce sufficient acetyl CoA to saturate the choline acetylase, provided only that an initial period for the mobilization of this system is allowed.

COHEN (1956) has recently commented that despite all the improvements in the reaction mixture, and the use of special methods for providing acetyl CoA, the values for brain choline acetylase activity are not higher than those indicated in the earliest studies 9 or 10 years ago. This is clearly not the case with rabbit brain extracts which yielded 2·0 to 2·2 mg ACh/g acetone powder per hr in studies by NACHMANSOHN and BERMAN in 1946, as contrasted to values of about 4 mg obtained by the various methods used in this study. This latter value is in excellent agreement with the value of 4·2 mg obtained by HEBB (1955) using rabbit brain extracts supplemented with the acetyl CoA forming enzymes of pigeon liver. HEBB (1955) showed also that the activity of the choline acetylase of a number of tissues from mammals and birds was invariably estimated higher when supplemented with pigeon liver extracts for the formation of acetyl CoA; furthermore, the difference was greatest with the most active preparations (chicken and pigeon retinas). It seems likely that in highly active tissues such as avian retinas, blowfly heads and squid head ganglia, the activity of the endogenous acetyl CoA forming system is incommensurate with the activity of the choline acetylase, so that special methods for ensuring an adequate

supply of acetyl CoA must be used to reveal the optimal activity of the enzyme. With tissues of relatively low activity, such as rabbit brain, the activity of the endogenous acetyl CoA forming system is more nearly balanced with the activity of the choline acetylase so that, as shown in this study, after a short initial period for the formation of acetyl CoA, ACh was synthesized at a linear rate for considerable periods of time.

Our results confirm the observation of BERMAN *et al.* (1953) that chemically-produced acetyl CoA is less effective than enzymically-produced acetyl CoA as substrate for choline acetylase. REISBERG (1955) (*née* BERMAN) has given a more detailed account of this anomaly. The discrepancy appears to be general, since they used a partially-purified preparation of squid head ganglia and we used a crude extract of rabbit brain. REISBERG (1955) also showed with her purified preparation, as we have with brain extracts, that the activity of choline acetylase was linear with time when provided with an enzymic system for the formation of acetyl CoA, but that the activity decreased with time when chemically-produced acetyl CoA was the sole source of this substrate. REISBERG suggests that the difference results from the inhibitory effect of free CoA produced during the reaction, and that this effect is prevented in the complete enzymic system by the reacetylation of CoA freed by the transfer of acetyl groups from acetyl CoA. Some reservation about this explanation must be expressed in view of our finding that the addition of ATP to provide the energy for reacetylation of CoA failed to improve the rate of ACh synthesis of brain extracts with chemically-produced acetyl CoA.

In a further effort to investigate this anomaly we obtained data on the relation between choline acetylase activity and acetyl CoA concentration, to enable calculation of the maximal rate of ACh synthesis. These data also enable calculation of the Michaelis-Menten constant for the choline acetylase of rabbit brain extracts with respect to acetyl CoA. Our value of $2{\cdot}2 \times 10^{-5}$ M indicates a high affinity of the enzyme for its substrate, acetyl CoA, and differs markedly from the only other published value of about 5×10^{-3} M obtained by BERMAN *et al.* (1953) with their purified preparation of squid head ganglia. Our preparation of acetyl CoA may have been purer than that used by BERMAN *et al.* since it was separated chromatographically from the acetylating mixture. In contrast to their result with the purified enzyme, we achieved nearly complete enzyme saturation within the range of acetyl CoA concentrations used. However, the main point of this experiment was that the calculated maximal activity of the choline acetylase of brain extracts provided with acetyl CoA itself still failed to exceed, and was in fact substantially less than the value of 4 mg ACh/g acetone powder per hr obtained with the various other methods used in this study.

SUMMARY

(1) The endogenous formation of acetyl CoA was shown to be rate-limiting for the synthesis of ACh by the choline acetylase of rabbit brain extracts.

(2) When an induction period for the formation of acetyl CoA was provided, the activity of choline acetylase was shown to be linear with time and enzyme concentration. The rate of ACh synthesis was about 4 mg ACh/g acetone powder per hr.

(3) By using several other methods for the enzymic formation of acetyl CoA, this rate was confirmed as maximal for the choline acetylase of brain extracts.

(4) Acetyl CoA itself, produced chemically, was only about half as effective as

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enzymically-produced acetyl CoA. From the relation between choline acetylase activity and acetyl CoA concentration, the calculated maximal rate of ACh synthesis was 2·3 mg ACh/g powder per hr and the Michaelis-Menten constant for the enzyme was $2{\cdot}2 \times 10^{-5}$ M-acetyl CoA.

Acknowledgements—The author wishes to thank Sir CHARLES HARINGTON for providing the facilities which enabled him to carry out this work;. Dr. W. FELDBERG for advice and encouragement; Mr. S. E. LEWIS of the Pest Infestation Laboratory, Slough, for assistance with the preparation of acetyl CoA, and Dr. C. O. HEBB for a supply of the pigeon liver extract.

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Journal of Neurochemistry, 1958, Vol. 2, pp. 128–137. Pergamon Press Ltd., London

THE LEVELS OF SOME FREE NITROGEN-CONTAINING PHOSPHATE ESTERS IN NERVOUS TISSUE

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(*Received* 16 *July* 1957)

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IN a previous study on the levels of free amino acids in nervous tissue (PORCELLATI and THOMPSON, 1957) evidence was obtained of the presence in aqueous alcoholic extracts of these tissues of a number of phosphorus-containing compounds, of which two were provisionally identified as phosphorylethanolamine and phosphorylserine. In view of the high content of phospholipids in the nervous system it was therefore decided to undertake a more detailed study of these 'lipid phosphate esters' (i.e. of the various mono- and di-phosphate esters that occur in the lecithins, kephalins and sphingomyelins) both in normal nervous tissue and in peripheral nerve undergoing Wallerian degeneration.

Various reports have already appeared on the detection of certain compounds of this type in brain; thus, phosphorylethanolamine has been identified by AWAPARA, LANDUA and FUERST (1950), ANSELL and DAWSON (1951), WALKER (1952), TALLAN, MOORE and STEIN (1954) and ROBERTS (1956); phosphorylcholine by DAWSON (1955*a*); glycerylphosphorylcholine by DAWSON (1955*b*); and glycerylphosphorylethanolamine by WALKER (1952), ANSELL and NORMAN (1953), TALLAN *et al.* (1954) and ROBERTS (1956). The latter worker has also provisionally identified a ninhydrin-reacting compound in brain as phosphorylserine.

No reports, however, have been found on the levels of these compounds in spinal cord or peripheral nerve. In order to obtain a more general picture, quantitative estimation of these compounds has been carried out in rat brain and hen brain, spinal cord and peripheral nerve. To obtain sufficient amounts of these phosphorus compounds for identification and quantitative estimation it was necessary, in the case of those compounds occurring only in low concentrations, to use relatively large amounts of nervous tissue for paper chromatographic analysis, and therefore to remove cations from the extracts by treatment with an ion-exchange resin, and orthophosphate by precipitation with barium, before applying to paper. After chromatographic separation, the phosphorus compounds were detected by the molybdate method of HANES and ISHERWOOD (1949) or by the ferric chloride-sulphosalicylic acid method of WADE and MORGAN (1953); they were then identified by the use of markers, or by hydrolysis of the eluted spots followed by chromatography and identification of the hydrolysis products.

EXPERIMENTAL

Materials. White rats, weighing between 150–250 g, and hens of mixed breed, weighing between 1 and 2 kg, were used. In the nerve transection experiments the right sciatic nerve was cut under

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nembutal and ether anaesthesia as previously described (PORCELLATI and THOMPSON, 1957). After killing the animals by decapitation, the tissues under investigation (brain, spinal cord and sciatic nerve) were quickly excised and immediately frozen on crushed ice in a deep-freeze.

Preparation of tissue extracts for paper chromatography. Tissue extracts for paper chromatography were prepared by homogenizing in 75% (v/v) ethanol, followed by evaporation of the centrifuged supernatant almost to dryness at temperatures not exceeding 50°C, as described by PORCELLATI and THOMPSON (1957). The volume of the extract used for chromatography was usually equivalent to 100–200 mg of fresh tissue.

Preparation of tissue extracts for ion-exchange and baryta purification. Frozen rat or hen brain (5–10 g) or frozen hen spinal cord (3–5 g) was homogenized in 2–3 vol. of ice-cold, distilled water. Proteins were precipitated by pouring into boiling water and maintaining at 100° for 1 min after bringing to pH 4·8–5·0 by addition of 0·5 N-HCl. The mixture was then filtered, and the precipitated protein washed twice with hot (70–80°) distilled water.

The clear, colourless filtrate, together with the washings, was concentrated under reduced pressure to about 10 ml, and then dialysed in the cold (0–4°) for two successive 12 hr periods against successive 100 ml volumes of distilled water, with continuous agitation by a magnetic stirrer; at the end of this time a sample, corresponding to more than 2 g of fresh tissue, of the fluid in the inner compartment was concentrated and found to contain only traces of glutamic and aspartic acids, and no other amino acids, when tested chromatographically (paper).

The dialysate (about 200 ml) was then evaporated under reduced pressure to about 6 ml, brought to pH 4·0–4·5 with 0·1 N-HCl, and passed through a column (38 × 1·3 cm) of Zeo-Karb 225 (Na form, 60–80 mesh—The Permutit Co. Ltd.), the rate of flow being about 18 ml/hr. The column was then washed with 70 ml H_2O, and the combined effluents pooled. A 2% aqueous solution of $Ba(OH)_2$ was added dropwise to the effluent until no further precipitation occurred on addition of a further drop to the centrifuged supernatant, the final pH being about 8·0–8·5. The Ba precipitate was centrifuged, washed once with a small quantity of water, centrifuged again, and the washed precipitate and supernatants collected separately.

For identification of the substances precipitated by the $Ba(OH)_2$, the washed precipitate was suspended in 1–1·5 ml distilled water; about 1 g Zeo-Karb 225 (Na form) was then added, and the mixture shaken mechanically for 20 min. After centrifuging for 5 min at 3000 g, samples of the clear supernatant were applied to paper for chromatography.

For identification of the Ba-soluble components, the supernatant obtained above was concentrated to a small volume under reduced pressure, and either electrolytically desalted, or partially freed from excess of $Ba(OH)_2$ by passing a stream of CO_2 through the solution followed by centrifugation. After these procedures samples were applied to paper for chromatography.

The volume of the final supernatant, or of the supernatant derived from the barium precipitate, used for chromatography was usually equivalent to 1 g of fresh tissue.

Chromatographic procedure. Paper chromatography for amino compounds, followed by detection with ninhydrin, was carried out as previously described (PORCELLATI and THOMPSON, 1957).

For detection of phosphorus-containing compounds, two-dimensional chromatograms were prepared on Whatman No. 1 and no. 541 papers. It was found necessary to wash the latter paper for 12 hr in 0·1 N-HCl, followed by 24 hr in running tap-water and 12 hr in distilled water, subsequently drying at room temperature; the Whatman No. 1 papers were used only when the method of WADE and MORGAN (1953) was applied for the detection of the separated phosphate esters, and were not washed.

The following solvent mixtures were used in the detection of phosphate esters:

(1) *n*-Propanol, ammonia, water (60 : 30 : 10 v/v/v) (HANES and ISHERWOOD, 1949).

(2) *n*-Propanol, water (80/20 v/v).

(3) Methyl ethyl ketone. methyl cellosolve, 20% acetic acid (40/15/30 v/v/v).

(4) Phenol, water (4 : 1 w/w), with NaCN (0·1 g) placed in a beaker in the tank to prevent oxidation of the phenol (DENT, 1948).

Ethylene diaminetetra-acetic acid (EDTA, 7·5 mg/100 ml) was dissolved in each of the solvent mixtures.

At the end of each run the solvents were allowed to evaporate from the papers at room temperature, 5–6 hr being sufficient for solvents (1), (2) and (3), although 2 days were allowed for the papers after running in phenol, which was in each case used as the second solvent.

Detection of P-containing compounds. P-containing compounds were detected on the chromatograms by the method of HANES and ISHERWOOD (1949), as modified by BANDURSKI and AXELROD (1951), or by the method of WADE and MORGAN (1953).

Using the first method, the sprayed papers were dried in an oven at 80° for 1–2 min, and then exposed to ultraviolet light for 10–15 min, this long exposure being necessary since certain of the phosphate esters present, notably phosphorylcholine, are relatively resistant to hydrolysis. Under these conditions the background paper remains almost colourless. The preliminary washing of the paper, together with the presence of EDTA, was found to prevent 'tailing' of the spots, and thereby to improve quantitative determination.

With the method of WADE and MORGAN (1953), it was found that the use of 2% sulphosalicylic acid gave better colour contrasts than the 1% solution originally recommended.

Identification of P-containing compounds. After determining the positions of the P-containing compounds as described above, identification was carried out by:—

(1) Comparison of the R_F of the unknown 'spot' with that of known phosphate esters. In some experiments the markers were run separately, and in others they were added individually to the tissue extracts before chromatography. This latter method was found to be more satisfactory for identification, since the R_F values of the pure compounds were mostly somewhat lower when run in the presence of the tissue extract.

(2) Hydrolysis of the unknown 'spots' after elution with water, followed by concentration and chromatography in order to identify the hydrolysis products. In the case of phosphorylcholine and glycerylphosphorylcholine the presence of choline was identified by the periodate method of APPLETON *et al.* (1953) as modified by WEBSTER (1956).

Estimation of P-containing compounds. After identification of the P-containing compounds as described above, the areas of paper carrying them were cut out from the chromatograms and digested with 1 ml 60% (w/v) perchloric acid and a few drops of 30% hydrogen peroxide. Phosphorus was estimated in the whole digest by the method of ERNSTER *et al.* (1950), as modified by STRICKLAND *et al.* (1956). The washed Whatman No. 541 papers gave negligible blanks, amounting to less than 0·02 μg P/cm^2; Whatman No. 1 papers gave blanks of about 0·15 μg P/cm^2.

When present in sufficient amounts, phosphorylserine was also estimated by the ninhydrin method, as described by PORCELLATI and THOMPSON (1957). Owing to the low ninhydrin colour yield/mole given by phosphorylethanolamine and glycerylphosphorylethanolamine, these compounds were not estimated by this means.

Phosphorylcholine and glycerylphosphorylcholine were also estimated by determining the free choline by the method referred to above after hydrolysis of the water eluates of the 'spots' in 6N-HCl for 48 hr at 120° in sealed tubes. After hydrolysis the solution was evaporated to dryness, and the residue quantitatively taken up in distilled water for the colorimetric estimation.

Total acid-soluble phosphorus was determined by the method referred to above.

Marker substances. The following substances were used as markers : phosphorylserine (Ba salt), phosphorylethanolamine (Ba salt) and phosphorylcholine (Ca salt), all synthesized by Dr. G. R. WEBSTER in this laboratory; glycerylphosphorylcholine (Cd salt) (GPC) prepared by Dr. G. R. WEBSTER from ovolecithin; glycerylphosphorylethanolamine (GPE), kindly provided by Dr. R. M. C. DAWSON; sodium α-glycerophosphate (L. Light & Co.), choline chloride (B.D.H.) and ethanolamine (L. Light & Co.).

Where necessary, conversion to the sodium salt was carried out by shaking with Zeo-Karb 225 (Na form), before applying to the papers.

RESULTS

The levels of phosphorylserine, phosphorylethanolamine and phosphorylcholine in brain are sufficiently high to allow their identification in the extracts obtained from 100–200 mg samples of tissue. On the other hand, the levels of the glycerylphosphoryl derivatives are so low as to render necessary a preliminary purification and concentration of the extracts obtained from about 1 g of tissue by means of treatment with an ion-exchange resin and with baryta, as described above.

It was necessary therefore to determine whether significant amounts of any phosphate esters were removed together with the orthophosphate by the baryta

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treatment. On testing the re-dissolved barium precipitate it was found that in addition to a large orthophosphate spot, some phosphorylserine and also three other unidentified P-containing spots were present. Phosphorylethanolamine, phosphorylcholine, GPC and GPE were never found in the barium precipitate, although they were all found in the barium-soluble supernatant of brain, together with some unprecipitated phosphorylserine, and four other unidentified P-containing components, of which one, judged by its R_F values, is very probably glycerophosphate. The presence of some phosphorylserine in the barium precipitate was confirmed by the use of ninhydrin. The distribution of these various substances between the barium precipitate and its supernatant has been confirmed by subjecting a synthetic mixture of the pure substances listed above to resin and baryta treatment, followed by chromatography and identification.

In the different tissues studied, phosphorylserine, phosphorylethanolamine and phosphorylcholine were readily identified in rat and hen brain. GPC and GPE were only identified when extracts of larger amounts (about 1 g) of rat and hen brain were used. Extracts of hen and rat spinal cord or sciatic nerve (100–200 mg) showed the presence of phosphorylserine and phosphorylethanolamine, although phosphorylcholine was only occasionally found in traces in these tissues. GPE was identified in hen spinal cord only when concentrated extracts were examined. GPC was not found in either spinal cord or sciatic nerve.

The serine and ethanolamine derivatives were identified by comparison of their R_F values with those of the added markers, and also by finding the expected hydrolysis products by paper chromatography after hydrolysis of the eluted spots. The choline derivatives were identified by R_F values, and by the detection of free choline by the periodate reaction in the hydrolysate. In addition the ninhydrin colour intensity/P content or choline/P ratios of the unknown substances were found to agree satisfactorily with those of the pure tracers.

Before proceeding to the quantitative estimation of these various phosphate esters on the paper chromatograms, some recovery experiments were carried out in which the pure markers were applied to the papers, detected by the HANES and ISHERWOOD method after chromatography, and then eluted, digested and submitted to P analysis, in order to test the adequacy of the methods used. The results of these preliminary experiments, which were carried out in duplicate, are shown in Table 1. It will be seen that while good recovery of phosphorylserine and phosphorylethanolamine was obtained, only about 70% of the phosphorylcholine was estimated. The low recovery of this latter compound may have been due to incomplete detection by the molybdate reactions of the whole area covered by the compound, since phosphorylcholine is only hydrolysed with difficulty; this view is confirmed by the fact that when P analysis was carried out on a spot located by the method of WADE and MORGAN, 85% recovery was obtained.

Experiments in which phosphorylserine and phosphorylethanolamine were added to brain extracts yielded 92% and 95% recovery respectively, as determined by P estimation of the eluted spots. No recovery experiments were carried out for the procedure involving barium precipitation; however, the results reported later for phosphorylcholine have shown good agreement between the choline analysis carried out on the barium supernatant and the P estimation carried out on the eluates from the spots on the direct chromatograms.

TABLE 1.—RECOVERIES OF SOME PHOSPHATE ESTERS AFTER CHROMATOGRAPHIC SEPARATION ON PAPER
(Expressed as μg P applied to paper and estimated in the eluate derived from the excised spot)

	Phosphoryl-serine	Phosphoryl-ethanolamine	Phosphoryl-choline
μg applied to paper	3·88	4·40	4·40
μg found	3·45	3·60	3·08
Percentage recovery	88%	84%	70%

Quantitative estimations of the amounts of phosphorylserine, phosphorylethanolamine and phosphorylcholine in the tissue extracts were next carried out by phosphorus determination of the cut-out areas of paper carrying the respective spots, after location by the method of HANES and ISHERWOOD. The results of these estimations are shown in Table 2. It will be noticed that while brain, spinal cord and sciatic nerve contains approximately the same levels of phosphorylserine, the concentration of phosphorylethanolamine is very much less in peripheral nerve than in brain.

TABLE 2.—MEAN LEVELS OF PHOSPHORYLSERINE, PHOSPHORYLETHANOLAMINE AND PHOSPHORYLCHOLINE IN NERVOUS TISSUE
(Expressed as mg/100 g wet wt).

	Rat brain (5)*	Hen brain (5)*	Hen spinal cord (13)*	Hen sciatic nerve (23)*
Phosphorylserine	4·0 (3·4–4·6)†	4·3 (3·9–5·1)†	5·5 ± 0·32	3·9 ± 0·30
Phosphorylethanolamine	14·1 (13·1–14·8)†	13·8 (7·4–16·0)†	6·8 ± 0·22	2·7 ± 0·23
Phosphorylcholine	7·1 (4·5–8·3)†	9·2 (7·1–12·1)†	—	—
Ratio $\frac{\text{Phosphorylethanolamine}}{\text{Phosphorylserine}}$	3·5	3·2	1·2	0·7

* No. of experiments.
† Range of values obtained.

The phosphorylcholine in extracts of hen brain has also been estimated by determination of the free choline, by the method of APPLETON *et al.* (1953), in water eluates of the phosphorylcholine spot on chromatograms prepared from the barium supernatant corresponding to 3 g of fresh tissue. The mean level of phosphorylcholine in six experiments, determined by this method, was 10·8 mg/100 g wet wt (range 8·6–13·1 mg/100g), which agrees well with the mean level of 9·2 mg/100 g as determined by phosphorus analysis.

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An estimate has also been made of the GPC content of hen brain. Since the level of this compound in brain is very low it was necessary to use the water eluates obtained from the GPC spots on four chromatograms prepared from the barium supernatant, corresponding in all to 4 g of fresh tissue. Four such experiments were carried out; in the first of these the eluate was hydrolysed in 6 N-HCl at 120° for 48 hr in sealed tubes. Subsequently it was found that heating for 1 hr in 6 N-HCl at 100°C under a reflux condenser gave complete hydrolysis, and these conditions were therefore used in the other three experiments. The mean value of GPC found was 0·69 mg/100 g wet wt (range 0·57–0·89).

Effects of nerve section

In the experiments on the effects of nerve section, extracts were prepared from the sectioned sciatic nerve and from the contralateral normal nerve of the same animal (hen) at intervals of from 4 to 28 days after transection, two hens being killed at each of the days studied. Phosphorylserine and phosphorylethanolamine were determined in these extracts by estimation of P in the cut-out areas of paper after location by the method of HANES and ISHERWOOD. The level of total acid-soluble phosphorus was also determined in the supernatants of homogenates of short lengths of the nerves in 10% trichloroacetic acid. The results of these experiments with degenerating nerves are shown in Table 3.

TABLE 3.—LEVELS OF PHOSPHORYLSERINE, PHOSPHORYLETHANOLAMINE AND TOTAL ACID-SOLUBLE PHOSPHORUS IN HEN SCIATIC NERVE AT INTERVALS AFTER NERVE SECTION.
(Expressed as μg/g wet wt. of nerve; mean of 2 experiments for each time after nerve section).

	Controls*	Days after section						
		4	8	13	15	18	23	28
Phosphorylserine	39	35	37	43	74	73	66	55
Phosphorylethanolamine	27	29	26	30	26	31	51	42
Total acid-soluble P	425	410	505	590	570	655	525	480

* Mean of 23 birds for phosphorylserine and phosphorylethanolamine estimations, and of 8 birds for total acid-soluble P estimations.

It will be seen that the levels of both phosphorylserine and phosphorylethanolamine undergo an increase during nerve degeneration, although the time relationships of the changes appear to differ in the two compounds, the maximum increase for phosphorylserine being at the fifteenth day, whereas the maximum for phosphorylethanolamine is not reached until the twenty-third day. As has been previously shown by LOGAN *et al.* (1952), an increase is also seen in the level of total acid-soluble phosphorus. Table 4 shows the changes in the content of these P derivatives in degenerating nerve, expressed as a percentage of the level in the contralateral normal nerve of the same animal.

TABLE 4.—CHANGES IN CONTENT OF PHOSPHORYLSERINE, PHOSPHORYLETHANOLAMINE AND TOTAL ACID-SOLUBLE PHOSPHORUS OF HEN SCIATIC NERVE AFTER TRANSECTION
(Results expressed as percentage change in sectioned nerve, compared with level in normal nerve of same bird; average of 2 experiments for each day after transection)

	Days after transection						
	4	8	13	15	18	23	28
Phosphorylserine	+1	+19	+78	+132	+120	+85	+63
Phosphorylethanolamine	+5	−10	−7	+5	+28	+72	+40
Total acid-soluble P	−5	+13	+31	+36	+82	+18	+17

DISCUSSION

The results described above present a general picture of the levels of phosphorylethanolamine, phosphorylserine and phosphorylcholine in the brain, spinal cord and peripheral nerve of the hen.

The level of phosphorylethanolamine found in rat brain, which agrees closely with that in hen brain, is also in good agreement with the mean values of 12 mg/100 g given by AWAPARA *et al.* (1950) and 9 mg/100 g given by ANSELL and DAWSON (1951) although somewhat lower than the value of 22 mg/100 g found by ANSELL and RICHTER (1954). In cat brain, TALLAN *et al.* (1954), using ion-exchange chromatography, found the considerably higher level of 41·9 mg/100 g. The levels of phosphorylcholine found in rat and hen brain correspond closely with the level of 8·1 mg/100 g found by DAWSON (1955*a*).

Phosphorylserine does not so far appear to have been definitely identified in tissue extracts, although ROBERTS (1956) suggested that an unidentified spot found earlier by him in chromatograms of brain might be due to the presence of this compound. Examination of the published reproductions of ninhydrin-developed chromatograms of other workers also reveals the presence of a faint spot having the approximate R_F found by us. Identification of this compound has been based on (i) the R_F values of the spot in four different solvent mixtures, as compared with the R_F of added phosphorylserine, (ii) the presence of P in the compound, (iii) the ratio of the intensity of the ninhydrin colour to the P content showed good agreement as between the unknown substance obtained from the barium supernatant and pure phosphorylserine, (iv) the chromatographic behaviour of acid hydrolysates of the compound: hydrolysis of the water eluates of the spot obtained from the barium supernatant was carried out by heating in 6 N-HCl for 48 hr at 100°; paper chromatography of the concentrated hydrolysate showed only spots of serine and orthophosphate.

As far as we are aware, the levels of these compounds in spinal cord or sciatic nerve do not appear to have been described. Although phosphorylcholine is present only in traces in these tissues, relatively large amounts of phosphorylserine and phosphorylethanolamine are present. The finding that the sciatic nerve contains about as much phosphorylserine as brain, whereas it contains only one fifth as much

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phosphorylethanolamine is of interest in view of BRANTE's (1949) statement that phosphatidylserine is a myelin lipid.

It will be seen also that the changes in the levels of these two compounds in degenerating peripheral nerve also show striking differences. Thus, the greatest increase in the level of free phosphorylserine occurs at about 15 days after transection, which is the time at which the free amino acids also show their greatest increase (PORCELLATI and THOMPSON, 1957), whereas a rise in the amount of free phosphorylethanolamine is not seen until after this time, the maximum being reached only at

TABLE 5.—R_F VALUES OF PHOSPHORUS-CONTAINING COMPOUNDS PRESENT IN BRAIN EXTRACTS

	Whatman No. 541 paper. Spots located by method of HANES and ISHERWOOD (1949)				Whatman No. 1 paper. Spots located by method of WADE and MORGAN (1953)			
	Solvent				Solvent			
	A	B	C	D	A	B	C	D
Phosphorylserine	0·05	0	0·02	0·29	0·05	0·01	0·02	0·28
X_1	0·10	—	0·15	—	0·10	—	0·13	—
X_2	0·11	0·16	—	—	—	—	—	—
Orthophosphate	0·12	0·04	0·08	—	0·12	0·06	0·08	—
Glycerophosphate	0·15	0·04	0·17	—	—	—	—	—
X_3	0·20	—	0·02	—	—	—	—	—
Phosphorylethanolamine	0·23	0·02	0·11	0·37	0·27	0·04	0·12	0·37
X_4	0·33	0·05	0·10	—	0·37	0·07	0·11	—
X_5	0·43	0·06	—	—	0·46	0·08	—	—
Glycerylphosphorylethanolamine	0·59	—	0·51	—	0·61	0·08	0·45	—
Phosphorylcholine	0·91	—	0·18	0·44	0·90	0·03	0·20	—
Glycerylphosphorylcholine	0·95	—	0·51	—	0·93	0·08	0·46	—

A = Phenol, water (4 : 1 w/w)
B = *n*-Propanol, water (80 : 20 v/v)
C = *n*-Propanol, ammonia, water (60 : 30 : 10, v/v/v)
D = Methyl ethyl ketone, methyl cellosolve, 20 per cent acetic acid (40 : 15 : 30, v/v/v).

about 23 days. In connection with the changes in free phosphorylserine and phosphorylethanolamine in the degenerating nerves it is of interest to recall that JOHNSON *et al.* (1949) showed that in degenerating cat nerve a marked fall in the kephalin fraction occurs after 16 days, although at 8 days the level is unchanged.

In addition to the identified compounds described above, a number of other P-containing spots were also regularly seen on the chromatograms. The R_F values of these various compounds, together with those of orthophosphate and glycerophosphate, in the different solvent mixtures are shown in Table 5. It must be stressed again that the values given for the identified compounds differ in most cases from those of the pure compounds in aqueous solution; for this reason, therefore, identification has in every case been checked by adding the marker substance to the tissue

extract, rather than by relying on comparison with the R_F of the pure substance in aqueous solution.

It will be seen that five unidentified P-containing substances, X_1, X_2, X_3, X_4, and X_5, have been detected. Substances X_1 and X_3 are only present in trace amounts, and were never detected when propanol-water was used as the first solvent; their presence when propanol-ammonia is used as the first solvent suggests that they are formed by the decomposition of a labile P-ester in the strongly alkaline solvent mixture; a similar decomposition product has been reported by DAWSON (1955*a*) in chromatograms of rat tissue in propanol-ammonia.

Substances X_4 and X_5 are present in relatively large amounts in brain, and X_4 has also always been detected in extracts of spinal cord and sciatic nerve. X_4, estimated by its P-content, shows a striking increase in degenerating sciatic nerve, rising from a value of 12·3 μg P/g (mean of eight estimations) to values of 15·6, 29·9 and 38·8 μg P/g on the eighth, thirteenth and eighteenth days respectively.

Although no estimations of glycerophosphate have been carried out in degenerating nerve, visual inspection of the P spots indicated that this compound also was undergoing an increase during the first 23 days of Wallerian degeneration.

The spot X_2 is only seen in chromatograms developed by the acid molybdate method of HANES and ISHERWOOD (1949), and then only becomes apparent if the developed chromatogram is left for 24 hr at room temperature. The intensity of this spot also undergoes an increase in degenerating nerve.

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SUMMARY

(1) A study has been made of the levels of a number of nitrogen-containing phosphate esters in nervous tissue.

(2) The presence of free phosphorylethanolamine, phosphorylcholine, glycerylphosphorylethanolamine and glycerylphosphorylcholine in brain has been confirmed.

(3) The presence of free phosphorylserine in brain, spinal cord and sciatic nerve has been demonstrated, and phosphorylethanolamine has also been detected in spinal cord and sciatic nerve.

(4) The levels of phosphorylserine and phosphorylethanolamine in degenerating sciatic nerve have been determined up to 28 days after nerve section. Each of these compounds was found to undergo an increase, the maximum level being reached at about 15 and 23 days respectively.

(5) A number of other unidentified P-containing spots have also been detected on the chromatograms, and one of these also undergoes a striking increase in degenerating nerve.

Acknowledgements—My thanks are due to Professor R. H. S. THOMPSON for suggesting this problem and for his help and advice, to Drs. R. W. R. BAKER and G. R. WEBSTER for many helpful discussions, and to the British Council for a Scholarship. I am also grateful to Drs. G. R. WEBSTER and R. M. C. DAWSON for the gifts of pure compounds, and to Miss MONICA HOVEY for technical assistance. The sectioning of hen nerves was kindly carried out by Professor R. H. S. THOMPSON.

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INFRARED SPECTROSCOPY OF NERVOUS TISSUE*†

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(*Received* 4 *May* 1957)

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SOME years ago PAULING suggested that field resonance and the hydrogen bond should play a part in such physiological phenomena as muscle contraction and transmission of impulses in nerve and brain. 'A conjugated system provides the only way of transmitting an effect from one end to another of a long molecule—and the H bond is the only strong and directed intermolecular interaction which can come into operation quickly' (PAULING, 1945). Although biologists have been slow to use the physical method of infrared spectroscopy, this approach is essential in the light of these concepts.

The infrared spectrum can reveal many details concerning a molecule, such as size, shape, strength of bonding, etc. and such information may be important in the study of nervous function. It is the purpose of the present paper to discuss some initial problems and observations made in the process of obtaining infrared spectra of brain tissue, and to point out possible directions of future investigation.

MATERIAL AND METHODS

Three types of brain tissue preparations were used for obtaining spectral analysis: (1) tissue sections; (2) brain homogenate; (3) lyophilized brain.

The tissue was obtained from rabbit and cat in three forms: cerebral grey matter, cerebral white matter and mixed grey and white. All the grey matter so far used was of cortical origin. Some sections were extracted with solvents so that spectra of the residues could be obtained.

The tissue sections were prepared by a modification of the technique of ADAMSTONE and TAYLOR (1949) as used by SCHWARZ *et al.* (1951*b*). A block of tissue was removed and frozen sections (about 8 mm long and 3–5 mm wide) were cut 25 μ and 50 μ thick. The microtome knife was cooled with dry ice and the section taken up on the cold surface of a dry-ice-filled stainless steel scoop. The section was transferred with cold glass needles to a 1 inch square silver chloride plate pre-cooled on dry ice and allowed to thaw for an instant to squeeze out any air trapped between the section and the plate.

The plate with the section firmly attached was then placed in a vacuum desiccator containing silica gel or Drierite for dehydration. The desiccator was kept in an insulated box surrounded by dry ice during the pumping and allowed to come to room temperature under vaccum after pumping was stopped. A silica gel drying tube was inserted in the vacuum train. The desiccator was chilled before the plates were placed it in, and was sealed with Apiezon L (low temperature grease). The pumping time necessary for adequate dehydration varied with the efficiency of the pump. With a new Cenco Hyvac about 8 hr at 0·1 mm Hg sufficed. Pumping for too long a period results in a chalky white appearance which causes an increase in light scattering. Such preparations can be used only if mulling agents, such as Nujol, are employed.

The silver chloride plates on which the sections lay were stored in a desiccator and kept in the dark. The spectra of sections stored for several months did not appear to be significantly different from those of fresh preparations.

* This work was supported by a grant from the Office of Naval Research, N7-onr-39706 and N7o-onr-59503.

† Part of the preliminary phase of the work was carried out by Dr. B. J. FAX. Technical assistance was rendered by Mrs. B. T. STEWART and Mrs. C. S. RUBIN.

A second group of spectra were obtained with homogenized brain preparations for comparison with homogenate spectra reported by THOMAS *et al.* (1954). The tissues were homogenized in distilled water (3 g/5 ml) using a cold stainless steel pestle. The homogenates were prepared for spectral analysis by placing several drops of the homogenate on a silver chloride plate and evacuating in a vacuum desiccator containing silica gel.

The lyophilized tissue was prepared in the usual way and the powder resuspended in distilled water before following the procedure described above for the homogenates. The homogenate and lyophilized preparations were stored in the same way as the tissue sections.

Extractions were made by passing drops of the desired reagent over the section for a few minutes. Sections soaked in chloroform–methanol (2 : 1, v/v) and chloroform alone, for four days, gave qualitatively the same spectra as those sections soaked for a few minutes. Consequently, the latter procedure was adopted as routine.

The spectra were obtained using a Perkin–Elmer Model 112, single beam (double pass) spectrophotometer with NaCl optics. Specimens on the silver chloride plates were masked with drafting tape, leaving exposed only an area which was generally about the same size as that covered by the infrared beam. The radiation curve was obtained with a blank silver chloride plate. With the scattering and the low level of transmission found in most of the sections, it soon became apparent that usual procedures for recording energy curves for the sample and the blank had to be discarded. At usual gain levels (about 0·5 μv for full-scale deflection), deflections of the order of 40–50 per cent were noted on the recorder when the slit-widths were used that had been determined in advance with a blank plate. The band contours were very fuzzy, and weak bands or bands showing up as shoulders on stronger bands were lost. Increasing the slit width rapidly destroyed whatever resolution one had. Consequently, it was decided that the normal procedure be reversed and the radiation curve be recorded after the sample curve. A fairly high gain (about 0·2 μv for full-scale deflection) was employed, so that reasonable slit-widths could be used. The energy curve of the sample was recorded and the radiation curve recorded at the same slit-widths (to preserve resolution) but with variable gain such that the previously determined slit-widths gave full-scale deflection. Each time the gain was so adjusted, the deflection produced by a test signal of 0·1 μv was recorded. With this arrangement, the per cent transmittance at a given wave number is obtained as follows:

$$\frac{\text{Deflection of sample}}{\text{Deflection of radiation}} \times \frac{\text{Gain used for radiation}}{\text{Gain used for sample}}$$

The gain is expressed in mm of deflection per 0·1 μv. The per cent transmittance was plotted as a function of wave number.

In addition to the normal brain samples, cerebral tissue was removed from two nembutalized cats which had been breathing a mixture of 7% O_2 : 93% N_2 for four hours. The tissue was removed while the animals were still breathing this mixture.

RESULTS

The absorption spectra of the tissue sections, homogenate films, and films prepared from lyophilized tissue were all qualitatively similar. The positions of the absorption maxima agreed within the experimental error. Typical spectra are shown in Fig. 1, and the absorption maxima given in Table 1. Part of the apparent difference in position of the bands listed in Table 1 for the different preparations can be ascribed to variation in thickness of the specimen. Both the homogenized and lyophilized films were significantly thicker than the tissue sections. This difference plus the factor of too great thickness itself, makes it extremely difficult to obtain the exact position of the absorption maxima. Inasmuch as this problem has shown itself to be of prime importance, it is intended in future analyses to measure thickness with as great an accuracy as necessary.

A preliminary survey estimating intensities of the absorption bands was made using the methods of SCHWARZ *et al.* (1951), THOMAS *et al.* (1954*b*), and the base line technique (WRIGHT, 1941). With such limited data, none of these intensity measurements gave consistent results among the tissue sections. Attempts to carry

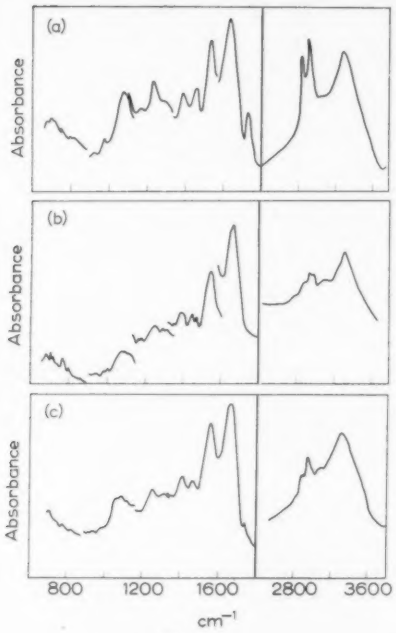

FIG. 1.
(a) Rabbit, cerebral cortex grey section. (b) Rabbit, cerebral cortex, grey section $CHCl_3$—MeOH extraction. (c) Rabbit, cerebral cortex, grey section $CHCl_3$ extraction.

TABLE 1.—TISSUE ABSORPTION BANDS (cm^{-1})

Band	Tissue sections	Tissue homogenates	Lyophilized Tissue
A	3291	3290	3305
B	3063	3061	3083
C	2918	2918	2928
D	2848	2843	2853
E	1735	1736	1742
F	1654	1646	1656
G	1543	1541	1547
H	1463	1456	1466
I	1388	1394	1393
J	1294	1289	1294
K	1235	1226	1231
L	1165	1159	1168
M	1068	1065	1069
N	970	970	971
O	924	923	926
P	873	872	869
Q	824	817	821
R	799	799	795
S	720	718	724
T	700	705	701

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out quantitative analysis of the data, as well as to develop new methods for such analyses, have not been pursued because of the present availability of only a single-beam spectrophotometer. Such efforts at quantitation are more properly carried out

TABLE 2.—POSITIONS OF ABSORPTION BANDS (cm^{-1}) IN TISSUE AND RESIDUES AFTER EXTRACTION

Band	Tissue*	Chloroform–methanol mixture	Chloroform	Methanol†
A	3291	3291	3291	3291
B	3063	3076	3070	3074
B_1	—	2951	2948	—
C	2918	2918	2915	2924
D	2848	2869	2854	2859
E	1735	1727	1731	1728
F	1654	1654	1656	1658
G	1543	1542	1542	1543
H	1463	1462	1466	1460
H_1	—	—	1456	—
H_2	—	1448	—	—
H_3	—	—	1436	—
I	1388	1393	1394	1398
I_1	—	1340	1344	1336
J	1294	1301	1298	1303
J_1	—	1275	—	—
K	1235	1238	1238	1238
L	1176	1162	1166	1168
L_1	—	1149	—	—
L_2	—	1103	1105	—
M	1068	1079	1080	1083
M_1	—	1056	1053	—
N	970	978	972	977
N_1	—	964	—	—
O	924	930	929	930
O_1	—	—	905	—
P	873	—	—	—
Q	824	835	837	—
R	799	800	808	810
R_1	—	—	777	780
R_2	—	752	—	745
R_3	—	—	—	731
S	720	—	—	726
T	700	699	700	700

* Tissue sections, homogenate and lyophilized films.
† Two sections from hypoxic cat.

with double-beam recording. With a single-beam instrument it is impossible to obtain a smooth, continuous curve throughout this entire spectral region. The difficulty here is primarily due to changes in slit-width from one region of the spectrum to another.

Some of the tissue sections were extracted with chloroform-methanol (2 : 1) mixture, methanol or chloroform. Typical spectra of the residues are shown in Fig. 1, and the positions of the band absorption maxima are given in Table 2. The

sections obtained from the animals breathing 7 per cent oxygen for a short time showed the same spectra as the sections from normal cats.

Most of the data are apparent from consideration of the tables. However, a certain number of observations can be specifically pointed out. In general, the spectra of the residue of the extractions contains more bands. The removal of material by extraction permits the appearance of weak absorption bands present in the tissue spectra. One band, *P*, disappears with all extractants and another, *M*, is shifted from 1068 to 1080 cm^{-1}. The spectra of the residues from the three extractions have a new band, *I*, near 1340 cm^{-1}. Chloroform–methanol and chloroform extracted residues have new bands at 2950, 1430–1456, 1105 and 1055 cm^{-1}. One band appears near 780 cm^{-1} after chloroform and methanol extractions and another near 750 cm^{-1} after chloroform–methanol and methanol extractions. Three new bands, 1275, 1149 and 964 cm^{-1} only appear after chloroform–methanol treatment; one, 905 cm^{-1}, after chloroform, and one, 731 cm^{-1}, after methanol. The band, *S*, appears only in the residue of the methanol extractions. Only one band, *Q*, is common to normal tissue and tissue extracted with chloroform and chloroform–methanol. This band is not seen after methanol extraction.

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DISCUSSION

The positions of the absorption bands in the spectra of tissues removed from the cerebral cortex and white matter underneath the cerebral cortex are identical. Tissues from both cat and rabbit have the same spectra. It has been reported (SCHWARZ, 1952; SCHWARZ *et al.*, 1951*a*, 1951*b*. 1952) that the spectra of tissues from the different areas of the brain were the same. They used dogs, rabbits, rats and human post mortem material and observed no changes in the spectra, when different methods of sacrificing the animals were used (air embolism, decapitation, etc.). THOMAS *et al.* (1954) found that the spectra of tissues removed from spinal cord and cerebellum of fowl and rat were identical qualitatively. This was also true for tissues from mouse (WOERNLEY, 1952) and human brains (SCHWARZ *et al.*, 1951*b*). COATES *et al.* (1953) studied the white and grey matter of rat hypothalamus with an infrared microscope and found additional bands in the limited region examined. The appearance of these bands may be due to the high resolution of the instrument. It should be noted that the 1300 cm^{-1} band appears as an inflection point in the spectra of the white matter and the 1163 cm^{-1} as an inflection point in the grey matter spectra. This probably indicates spectral differences which occur at various points in the tissue. WOERNLEY (1952) has found slight differences in the spectra of two fractions of mouse tissue cytoplasm.

These data indicate that similar spectra are obtained with tissues from the central nervous system of different species, after various methods of sacrificing the animals. The method of preparing tissue for spectral analysis has no effect on the number and positions of absorption bands. Homogenate films have the same spectra as tissue sections (Table 1 and THOMAS *et al.*, 1954). This has also been confirmed for tissue which has been lyophilized and prepared as a film (Table 1). However, although the positions of the absorption bands are invariant with the method of preparation, it should be expected that the absorptivities of the components contributing to the absorption would probably be altered with the different methods of preparation.

It would be desirable as an aid in the interpretation of changes which might occur

in spectra of tissue altered by some stress or drug to determine what chemical constituent has been altered in amount and/or molecular structure. Table 3 represents a compilation of the spectral characteristics of certain substances which have been shown or are presumed to exist in nervous tissue. This does *not* mean that the spectrum of nervous tissue is considered to be the sum of these findings but merely that such a spectrum must be related to them in some way or other. It should be noted that some spectra were obtained in solution. The positions of these bands are probably different from those observed in the spectra of the solids where such factors as H bonding and crystal structure alter the spectra. Generally, the positions of the bands were estimated from the published curves so that the exact position is not known. It is proposed to measure the spectra of these substances on our own instrument to eliminate the variations due to the different equipment. It should be acknowledged that these are only suggestive correlations. THOMAS *et al.* (1954) have suggested that band *E* is due to C=O in fatty acid. Bands (COLTHUP, 1950; RANDALL *et al.*, 1949) in this region have usually been assigned to C=O in the esters of fatty acids which are present in lipides of nervous tissue.

The resemblance between the spectra of nervous tissue and the spectra of its components becomes greater as the molecule becomes larger and more complicated. Hence, the spectra of cerebron (MISLOW, 1952) and lipoprotein (FREEMAN *et al.*, 1953) bear a close resemblance to the tissue spectra. BLOUT and MELLORS (1949) suggested a close correspondence between protein and tissue spectra. There are contributions from nucleic acid absorptions (Table 3) and perhaps the spectrum of a liponucleoprotein would most closely approximate to the spectrum of nervous tissue. It is apparent from Table 3 that no single assignment can be made for each band, since many constituents in the tissue have different groups with similar absorption bands.

Extraction of the tissue with various solvents provides a method of analysis for tissue constituents. An investigation of the residue and extractant is desirable. However, the spectrum of the residue itself may provide clues as to what changes may have occurred in tissues subjected to various stresses. The action of chloroform on the nerve fibre induces a reduction of the intensities of the bands at 2900, 1460, 1375, 1235 and 1080 cm^{-1} in the spectrum of the residual 'fibre.' The first three bands arise from CH vibrations and the last from C—O—C esters and would be expected to be diminished in intensity since these groups are present in lipides. The 'band near 1235 cm^{-1} appears to be polyamide rather than lipide' (BARER *et al.*, 1949). However, there are contributions to this absorption from the nucleic acids (BLOUT and FIELDS, 1948, 1949; FRASER and FRASER, 1951) (Table 4).

SCHWARZ *et al.* (1951*a*, 1951*b*, 1952) have examined the effect of various lipide solvents upon the spectra of nervous tissues. It is stated that the bands at 970 and 1075 cm^{-1} 'completely disappeared after extraction' with chloroform–ether mixture and the 1240 cm^{-1} band 'was greatly diminished' (SCHWARZ *et al.*, 1951*b*). However, a close examination of the published curves (Fig. 1, SCHWARZ *et al.*, 1951*b*) for the extraction of sections of rat brain cerebellum and white matter of the hippocampus reveals that these bands are present after extraction. The extraction of rat brain stem with this solvent 'greatly reduces the characteristic absorption bands .... in the 9–11 μ region' (SCHWARZ *et al.*, 1952), but not the amide vibrations (1550 cm^{-1}). This is in agreement with the observations made in the present investigation. Various lipide solvents (SCHWARZ *et al.*, 1952) were used as extractants, and ethanol had a

TABLE 3.—POSSIBLE ASSIGNMENTS FOR NERVE TISSUE ABSORPTION BANDS

Band (cm^{-1})	Group vibrations	Compounds			Lipides and other compounds
		Protein	Lipo-protein	Nucleic acid	
A–3291	N—H stretch, associated O—H stretch, associated (11)	12	15	8, 14	Sphingomyelin (3, 15, 20) Cerebroside (20) Lecithin (3, 15, 16, 19, 20) Acetal phospholipides (20)
B–3063	C—H stretch C—H associated with double bond (30) N—H stretch, associated (9)	12	15		Esters of long chain fatty acids (28)
C–2918	C—H stretch CH_2 antisymmetrical (29)		15		Sphingomyelin (15) Lecithin (3, 15) Cholesterol and cholesterol esters (9, 13) Fatty acids (29, 30)
D–2848	C—H stretch CH_2 symmetrical (29)	12	15		Cerebron (21) Sphingomyelin (15) Lecithin (3, 15, 16) Cholesterol and esters (15) Sphingosine (21) Fatty acids (29, 30)
E–1735	C=O stretch, esters (11, 23)		15		Lecithin (3, 15, 16, 20) Kephalin (4, 7, 20) Cholesterol esters (6, 15) Acetal phospholipides (20) Fatty acids esters (28–30)
F–1654	C=C stretch C=O stretch, proteins, amides C=N stretch N—H bending, amines (11) CONH amide I (23)	12	15		Cerebron (21) Sphingomyelin (15, 20, 27) Lecithin (16, 20) Kephalin (4) Glycolipides (27) Cholesterol (15) Unsaturated fatty acids, Esters (30)
G–1543	N—H bending (11) CONH amide II (23)	12	15		Cerebron (21) Sphingomyelin (15, 19, 27, 20) Cerebroside (20) Kephalin (4, 20) Acetal phospholipide (20) Glycolipides (27) Sphingosine (20, 21)

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TABLE 3 (*continued*)

Band (cm^{-1})	Group vibrations	Compounds			Lipides and other compounds
		Protein	Lipo-protein	Nucleic acid	
H–1463	C—H bending CH_2, CH_3	12	15	8	Cerebron (21) Lecithin (16, 19) Sphingosine (21) Cholesterol (13, 15) Long chain fatty acids, esters (29, 30)
I–1388	C—H bending CH_3		15	8	Cerebron (21) Sphingomyelin (15) Lecithin (3) Sphingosine (21) Cholesterol (13) Long chain fatty acids, esters (28)
J–1294	P═O stretch (6, 11) C—H bending in trans —C═C— (6)				Kephalin (4, 20) Cholesterol (5, 13)
K–1235	P═O stretch (6, 14) C—O—C stretch (23, 28)		15	8, 14	Cerebron (21) Sphingomyelin (15, 20) Lecithin (3, 15, 20) Kephalin (7) Sphingosine (20, 21) Cholesterol and esters (15) Long chain fatty acids, esters (29, 30)
L–1176	C—O stretch (28) C—O—C stretch, esters (3, 7, 23)		15		Cerebron (21) Kerebroside (20) Lecithin (3, 15, 16, 20) Kephalin (4, 7, 20) Cholesterol and cholesterol esters (13, 15) Long chain fatty acids, esters (28, 29)
M–1068	P—O—C stretch (6, 7) C—O—C stretch, sugar (14) esters (6) C—O stretch, alcohols (6) O—H bending (6)		15	8, 14, 24	Cerebron (21) Sphingomyelin (15, 20) Lecithin (3, 15, 20) Kephalin (4, 7) Glycolipides (27) Phospholipides (27) Sphingosine (20, 21) Cholesterol (5, 13, 15)

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TABLE 3 (*continued*)

Band (cm^{-1})	Group vibrations	Compounds			Lipides and other compounds
		Protein	Lipo-protein	Nucleic acid	
N–970	C—H bending in trans —C=C—		15	8, 14, 26, 25, 24	Cerbron (21) Sphingomyelin (15, 19, 20) Cerebroside (20) Lecithin (3, 15, 16, 20) Kephalin (4, 20) Sphingosine (20, 21) Cholesterol and esters (5, 13, 15)
					Unsaturated fatty acids (30) Glucose-1-phosphate (24)
O–924			15	DNA (8)	Sphingomyelin (15, 20) Lecithin (3, 15, 16, 20) Kephalin (4, 20) Cholesterol and esters (5, 13, 15) Acetal phospholipide (20) D-Glucose (24)
P–873			15	RNA (8)	Sphingomyelin (15, 20) Cerebroside (20) Lecithin (3, 15, 16, 20) *N*-lignocerysphingosine (20) Cholesterol (5, 13, 15) Glucose-1-phosphate, D-deoxyribose and D-ribose (24)
Q–824			15	RNA (8)	Lecithin (15, 16) Kephalin (4, 20) Acetal phospholipide (20) *N*-lignocerylsphingosine (20)
R–799	$(CH_3)_2$—C (6)				Lecithin (15) Kephalin (20) Cholesterol and esters (5, 13, 15) *N*-lignocerylsphingosine (20) D-ribose (24)
S–720	$-(CH_2)_i-$(11, 28) R—CH (28)		15	8	Cerebron (21) Sphingomyelin (20) Cerebroside (20) Lecithin (15, 20) Kephalin (4) Acetal phospholipide (20) Sphingosine (20, 21) Cholesterol ester (15) Long chain saturated fatty acids (28)
I–700	N—H bending out of plane (18)	2, 18	15		Cerebron (21) Long chain unsaturated fatty acids (30)

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TABLE 4.—POSSIBLE ASSIGNMENTS FOR ADDITIONAL ABSORPTION BANDS IN TISSUE RESIDUES FROM VARIOUS EXTRACTIONS

Band (cm^{-1})	Extraction* medium	Group vibrations	Compounds			Lipides and other compounds
			Proteins	Lipo-proteins	Nucleic acid	
B_1–2951	CM, Ch	C—H stretch antisymmetric, CH_3 (6)				Long chain fatty acids, Esters (29,30)
H_1–1456	Ch	C—H bending				Triolein (28)
H_2–1448	CM	C—H bending, CH_2 (28)	12			Long chain fatty acids, Esters (28)
H_3–1436	Ch	C—H bending, CH_2 adjacent to C=C (tentative) (30)				Cholesterol (13) Long chain unsaturated fatty acids (30)
I_1–1340	CM, Ch, M	C—H wagging (29)				Cerebron (21) Cholesterol (13) Long chain saturated fatty acids and esters (29)
J_1–1275	CM					Cholesterol (13)
L_1–1149	CM	C—O stretch (7)				Kephalin (7) Cholestrol (13)
L_2–1103	CM, Ch	C—O stretch (7)				Kephalin (7) Cholesterol (13) Triglycerides (28)
M_1–1056	CM, Ch	C—O—P stretch 7, 14		15	DNA(14)	Cerebron (20) Sphingomyelin (15) Lecithin (15) Kephalin (4, 7) Cholesterol (5, 13, 15, 27) Sphingosine (21)
N_1–964	CM	C=C trans (30)			8, 14	Cholesterol (5, 13) Triglycerides (28) Long chain unsaturated fatty acids (30)
O_1–905	Ch				RNA(8)	Cerebron (21)
R_1–777	Ch, M				DNA(8)	Sphingomyelin (15) Sphingosine (21)
R_2–752	CM, M	P—C, aliphatic (6)				Kephalin (4)
R_3–731	M				8	Cholesterol (13)

* CM—chloroform : methanol (2 : 1)
Ch—chloroform
M—methanol

greater effect than the chloroform–ether mixture. It reduced the intensity of the bands listed above. The nature of the substances extracted was not established. However, it may be suggested that ethanol may remove some lipoproteins, glycolipides, or sphingomyelin, whereas the chloroform–ether mixture does not.

In the present investigation there was an increase in the number of bands after extraction. The additional bands and possible correlations with known spectra are given in Table 4. These correlations indicate that complete extraction of the lipides has not occurred. It has been reported that all brain lipides are not removed after one hour extraction with hot chloroform–methanol (WAKSMAN *et al.*, 1954). KEILIG (1944) states that 24 hours are required for complete extraction of lipides from brain tissue even with hot reagents. In view of these results, it is not surprising that extraction at room temperature even for as long as four days or in SCHWARZ's experiments, 15–50 hours at 4° (SCHWARZ *et al.*, 1952) does not remove all lipides. The solvents may extract enough of the lipides so that weak bands such as 720 cm^{-1} disappear and other bands weaken. This would permit the appearance of other bands which were very weak or were part of the highly absorbing bands in the tissue spectra. The appearance or disappearance of bands would depend upon what the individual solvents would extract.

The examination of the spectra of both the residues and extractants should provide further information for the identification of the tissue absorption bands. This might be a valuable method of obtaining information in this area. Future work could be carried out with such extraction procedures not only with lipide solvents, but by step-by-step extractions of various types. A major consideration is the necessity to use extractants and conditions which induce minimal alterations in the normal structural orientation of the tissue components. However, the complexity of the material which is being dealt with gives rise to special problems of quantitation and interpretation. These problems can be partially simplified by correlating the infrared spectral data with those obtained by other methods—chemical, optic (e.g. ultraviolet), chromatographic, electrophoretic, ultracentrifugal, etc. The extracted materials—obtained by refined fractionation and extraction procedures—also need to be analysed in this integrated way. Such correlated infrared spectroscopy can yield a great deal of accurate, usable data relative to the fundamental chemical composition and structure of substances in various areas of the brain under different conditions. Using the same extraction procedures with tissues from stressed and control animals, any changes in the molecular structure of any substance could be determined.

SUMMARY

A procedure is described for obtaining the infrared spectra of frozen-dried tissue sections of brain. The spectra of homogenate film and lyophilized tissue film are identical with the spectra of sections. Analysis of the tissue which would permit quantitative distinction between cerebral grey matter and white matter is not possible with a single-beam spectrophotometer.

Extraction of the tissue sections at room temperature with lipide solvents, chloroform-methanol, chloroform, and methanol, does not remove all lipides as indicated by the spectra of the residues. The extracted residues have more absorption bands than the unextracted tissue sections. The appearance or disappearance of certain

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bands appear to be functions of the solvent used. This indicates that the extractants are removing different substances.

A comparison with spectra obtained by different investigators reveals that with present methods, similar spectra are obtained from different areas of the brain and from different species. Possible assignments of the spectra of tissue and extracted tissue residue are made from the spectra of brain components reported in the literature.

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STOFFWECHSELVORGÄNGE IM GEHIRN BEI NORMALER UND HERABGESETZTER KÖRPERTEMPERATUR UNTER ISCHÄMISCHER UND ANOXISCHER BELASTUNG*†

METABOLIC PROCESSES IN THE BRAIN AT NORMAL AND REDUCED TEMPERATURES AND UNDER ANOXIC AND ISCHAEMIC CONDITIONS

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(*Received* 12 *May* 1957)

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In einer früheren Mitteilung konnten THORN, PFLEIDERER, FROWEIN u. ROSS (1955) über erste Versuche berichten, die mit dem Ziel unternommen wurden, Beziehungen zwischen energieliefernden Umsetzungen und der Funktion des Zentralnervensystems zu finden.

In den Extrakten der mit flüssiger Luft eingefrorenen Gehirnen sollte eine möglichst grosse Anzahl von Metaboliten quantitativ erfasst werden. Zu diesem Zweck wurde unter anderem eine Reihe fermentativer Bestimmungsmethoden ausgearbeitet und für die quantitative Erfassung von Metaboliten in Gewebsextrakten routinemässig angewandt. Die Verwendung optischer Fermentteste bedeutet einen Gewinn an Spezifität und eine erhebliche Zeitersparnis.

Den Einfluss einer akuten Ischämie (plötzliche Unterbrechung des Gehirnkreislaufs) und einer Anoxie (Stickstoffbeatmung) auf den Organismus wollten wir ebenfalls durch die Registrierung von Elektroencephalogramm, Elektrocardiogramm und Blutdruck während der Versuche prüfen und gleichzeitig eine Kontrolle für die Reproduzierbarkeit unserer Tierversuche finden.

Das Untersuchungsprogramm war tierexperimentell und analytisch-chemisch recht umfangreich. Es waren Fehler und viele Verbesserungsmöglichkeiten darin enthalten. Wir haben deshalb nach weitgehender Überarbeitung und Verbesserung unserer Versuchstchnik die Ischämie—und Anoxieversuche bei normaler Körpertemperatur wiederholt und den Einfluss derselben Belastung an ausgekühlten Tieren bei 26°C geprüft, da die Herabsetzung der Reaktionsgeschwindigkeit für die Erfassung sehr rasch verlaufender Stoffwechselvorgänge vorteilhaft ist. In dieser Arbeit berichten wir über die Befunde an 170 Gehirnen.

Darüber hinaus wurden die Stoffwechseluntersuchungen auf Herz, Leber, Niere und Muskulatur ausgedehnt, so dass wir über gleichzeitig auftretende Veränderungen in diesen 5 Organen etwas aussagen können. Die Verbesserung unserer Registriertechnik und die Befunde an den aufgezählten Organen werden in folgenden Arbeiten abgehandelt (SCHOLL u. THORN, 1957; THORN *et al.*, 1957*a*, *b*, *c*).

* Kurze Mitteilung XX internat. Physiologen-Kongress, Bruessel, August (1956).

† Unser ergebener Dank für finanzielle und apparative Unterstützung gilt dem Ministerium für Wirtschaft und Verkehr, Düsseldorf, der Gesellschaft der Universität Köln und der Deutschen Forschungsgemeinschaft.

METHODIK

Abkürzungen:

ALD	Diphosphofructose-Aldolase
GDH	α-Glycerophosphat-Dehydrogenase
MDH	Milchsäure-Dehydrogenase
ox. Ferm.	Phosphoglycerinaldehyd-Dehydrogenase (oxydierendes Gärungsferment)
PK	Pyruvat-Kinase
Pü. I	Phosphatübertragendes Ferment
GLDH	Glutaminsäuredehydrogenase
TA_1	Glutamat-Pyruvat-Transaminase
ÄDH	Äpfelsäuredehydrogenase
TA_2	Asparat-Glutamat-Transaminase
ATP	Adenosintriphosphat
ADP	Adenosindiphosphat
DPN	Diphosphopyridinnucleotid
TPN	Triphosphopyridinnucleotid
PKr	Phosphokreatin
FDP	Fructosediphosphat
DAP	Dioxyacetonphosphat
PGA	Phosphoglycerinaldehyd
PBTS	Enolphosphobrenztraubensäure
BTS	Brenztraubensäure
MS	Milchsäure
EEG	Elektroencephalogramm
EKG	Elektrocardiogramm
ZNS	Zentralnervensystem
mmol/l.	Millimol/Liter

Normaltiere: Der Kopf des Tieres wird nach Entfernung der Schädeldecke mit dem freigelegten Gehirn *in situ* in flüssiger Luft eingefroren, anschliessend wird das Tier dekapitiert und das Gehirn aus dem gefrorenen Schädel herauspräpariert.

Nulltiere: Das freigelegte Gehirn wird mit einem auf −180°C gekühlten Löffel von Tieren ohne ischämische oder anoxische Belastung entnommen und in flüssige Luft geworfen.

Tierversuche (Einzelheiten THORN *et al.*, 1955)

An ausgewachsenen, narkotisierten Kaninchen (1,0–1,2 g Urethan/kg, lokal Novocain 2 Prozent) haben wir unter Schonung der Dura das Gehirn weitgehend freigelegt und bei normaler Körpertemperatur und Abkühlung auf 26°C Ischämie- und Anoxieversuche durchgeführt. Die akute Unterbrechung der Gehirndurchblutung (Ischämie) wurde mit Hilfe der von OPITZ u. THORN (1949) angegebenen Manschettenmethode erzeugt. Für die Anoxieversuche wurde ein Gasgemisch von 5,5 Prozent CO_2, 94,35 Prozent N, 0,15 Prozent O verwandt. Die Einwirkung von Stickstoffbeatmung und Kreislaufunterbrechung wurde bei normaler Körpertemperatur bis zu 15 min und an den abgekühlten Tieren bis zu 25 min verfolgt. Am Ende der jeweils geplanten Versuchszeit und bei den Nulltieren wurde das Gehirn mit einem auf −180°C gekühlten, scharfen Löffel entnommen und in 1–2 sec in die flüssige Luft geworfen. Da durch den Reiz der Herausnahme in der kurzen Zeit schon eindeutig messbare Veränderungen in der Metabolitkonzentration auftreten (THORN *et al.*, 1955), haben wir neben den Nulltieren bei einer Reihe von Tieren das Gehirn freigelegt und anschliessend den ganzen Kopf des Tieres eingefroren (Normaltier).

Aufarbeitung der Organe

Für die Enteiweissung der Organe wurde eine M/3 $HClO_4$-Lsg.benutzt, da man $HClO_4$ mit KOH neutralisieren und als $KClO_4$ fällen und abzentrifugieren kann. Vor dem Neutralisieren wurden einige ml entnommen und als saurer Extrakt für die Ammoniakbestimmung abgezweigt (THORN u. HEIMANN, 1957*a*). Es zeigte sich jedoch, dass selbst geringe Mengen $KClO_4$ in den Extrakten die geplanten fermentativen Bestimmungen von Alanin, Asparaginsäure und Glutaminsäure stören, da $KClO_4$ die Transaminasen (TA_1 und TA_2) hemmt. Wir mussten deshalb neben dem neutralisierten und sauren Extrakt aus einem Teil der Organe einen Kochsaft herstellen, an dem die fermentative Bestimmung der genannten Aminosäuren möglich war.

Chemische Methoden

In dieser Arbeit wird über die Veränderungen der Konzentration an Adenosintriphosphat (ATP), Adenosindiphosphat (ADP), Fructosediphosphat (FDP), Dioxyacetonphosphat (DAP), Brenztraubensäure (BTS), Alanin, Asparaginsäure, Glutaminsäure, Milchsäure (MS), anorganischem Phosphat, Phosphokreatin (PKr) berichtet.

Die Bestimmungen von MS (Prinzip: BARKER und SUMMERSON, 1941), anorganischem Phosphat (ERNSTER *et al.*, 1950) (Phosphomolybdatkomplex in *Iso*butanol-Benzol-Gemisch überführt und mit $SnCl_2$ reduziert), PKr (Hydrolyse des Extrakts 30 min 30°C in N-H_2SO_4 + Ammoniummolybdat mit anschliessender Phosphatbestimmung) erfolgte auf analytisch-chemischem Wege (Einzelheiten THORN *et al.*, 1955). Für die Erfassung der übrigen angeführten Metabolite benutzten wir spezifische optische Fermentteste, die bei PFLEIDERER *et al.* (1955*a*, *b*) und THORN *et al.* (1955) beschrieben sind. Alle Fermentteste sind auf die Ausnutzung der starken Absorptionsbande des reduzierten Diphosphopyridinnucleotids (340 mμ DPN red) aufgebaut. Zur Übersicht seien hier kurz die Metabolite mit den Fermenten angeführt, die für die Bestimmung benutzt werden:

Metabolite	*Fermente*
ATP (THORN *et al.*, 1955).	Phosphoglycerinaldehyd-Dehydrogenase (ox. Ferm.) + Phosphatübertragendes Ferment (PüI).
ADP (THORN *et al.*, 1955).	Pyruvatkinase (PK) + Milchsäuredehydrogenase (MDH).
DAP (THORN *et al.*, 1955).	α-Glycerophosphat-Dehydrogenase (GDH).
FDP (THORN *et al.*, 1955).	α-Glycerophosphat-Dehydrogenase (GDH) + Aldolase (ALD).
BTS (THORN *et al.*, 1955).	Milchsäure-Dehydrogenase (MDH).
Alanin (PFLEIDERER *et al.*, 1955*a*).	Glutamat-Pyruvat-Transaminase (TA_1) + Glutaminsäuredehydrogenase (GLDH).
Asparaginsäure (PFLEIDERER *et al.*, 1955*b*).	Asparat-Glutamat-Transaminase (TA_2) + Glutaminsäuredehydrogenase (GLDH).
Glutaminsäure (PFLEIDERER *et al.*, 1955*a*).	Glutamat-Pyruvat-Transaminase (TA_1) + Glutaminsäuredehydrogenase (GLDH).

Ein Vergleich von analytisch-chemisch gewonnenen Werten für die MS-Konzentration mit encymatischen Bestimmungen (PFLEIDERER u. DOSE, 1955*c*) wird von THORN *et al.* (1957*c*) publiziert.

ERGEBNISSE

Normaltiere, Nulltiere (Abb. 1–5, Tab. 1–3)

Aus Untersuchungen von KERR (1942), DAWSON u. RICHTER (1950), RICHTER u. DAWSON (1948), KRATZING (1953) und KRATZING u. NARAYANASWANI (1953) ist bekannt, dass mechanische oder elektrische Reizung von Gehirngewebe Metabolitveränderungen hervorruft. In unserer ersten Mitteilung (THORN *et al.*, 1955) konnten wir diese Beobachtung bestätigen und berichten, dass sich die Konzentrationen an anorg. Phosphat, PKr, ATP und FDP unter dem Einfluss der Herausnahme mit einem vorgekühlten Löffel (Nulltier) gegenüber dem in situ eingefrorenen Gehirn verändert. Die Versuche wurden mit verbesserter Versuchstechnik bei normaler Körpertemperatur und bei 26°C wiederholt; denn wenn es sich um echte Reaktionen handelte, mussten die Veränderungen bei Temperatursenkung kleiner werden. Die Ergebnisse sind in den Abb. 1,3 u. 5 und den Tab. 1 u. 2 wiedergegeben, allen Angaben liegt die Dimension Millimol/Liter (mmol/l.) zugrunde.

Das anorg. Phosphat (Abb. 1 u. 2) schnellt unter dem Reiz des Herauslöffelns im Mittel um 1,8 mmol/l. in 1–3 sec in die Höhe (2,53 auf 4,37), ein Wert, der mit unseren früheren Messungen übereinstimmt. Bei 26°C beträgt die mittlere Differenz 0,75 mmol/l. (2,44 auf 3,19), also nicht ganz die Hälfte des Anstiegs bei normaler Körpertemperatur.

Als 'labiles Phosphat' haben wir eine Fraktion bezeichnet, die unter 1 N-H_2SO_4 30 min im Wasserbad bei 30°C freigesetzt wird und in ihrer Bindungsfestigkeit dem

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TAB. 1.—METABOLITKONZENTRATIONEN IM GEHIRN VON NORMAL- UND NULLTIEREN, NACH ISCHÄMIE- UND ANOXIEVERSUCHEN BEI 37°C KÖRPERTEMPERATUR

Alle Angaben in 10^{-3} mol/l.

			Ischämie 37°c					Anoxie 37°c		
	Normaltiere	Nulltiere	2 min J.	5 min J.	9,15 min J.	10 min J.	15 min J.	5 min A.	10 min A.	15 min A.
Mich-säulre	1,58—2,66 (5) 2,2	1,6—2,82 (10) 2,145	7,4—10,5 (3) 9,13	15,6—18,1 (3) 16,5		15,5—26,6 (3) 21,9	18,3—26,5 (3) 23,7	13,5—16,1 (3) 15,0	19.2—25,0 (4) 22,2	25,0—26,6 (4) 25,1
anorg. P	1,97—3,14 (9) 2,53	3,30—4,80 (6) 4,37	4,93—5,4 (6) 5,2	7,0—9,45 (4) 8,5	8,2	9,05—9,7 (4) 9,3	10,6—11,75 (3) 11,25	5,5—8,95 (3) 7,65	8,4—11,9 (5) 10,12	11,1—13,4 (4) 11,95
PKr	2,88—3,66 (9) 3,26	2,03—3,8 (6) 2,47	0,42—0,67 (6) 0,59	(4) 0	0	(4) 0	(2) 0	0,74+0,685 (2) 0,712	(5) 0	(4) 0
FDP	0,028—0,051 (4) 0,0393	0,10—0,154 (5) 0,128	0,099—0,13 (5) 0,112	0,06—0,092 (3) 0,0702		0,027+0,042 (2) 0,0345	0,057+0,065 (2) 0,061	0,078+0,128 (2) 0,103	0,058—0,089 (4) 0,076	0,014—0,063 (3) 0,043
BTS	0,0125—0,0193 (4) 0,0158	0,0117—0,0416 (4) 0,0267	0,007—0,0141 (3) 0,01052	0,0127+0,0202 (2) 0,0165		0,0105+0,0117 (2) 0,0111	0—0,0114 (2) 0,0057	0,0149+0,015 (2) 0,01495	0—0,0165 (5) 0,0058	0,00344—0,0068 (3) 0,00535
DAP	0,0197—0,031 (4) 0,0242	0,0295—0,0441 (4) 0,03608	0,023—0,0338 (3) 0,02837	0,0161+0,0192 (2) 0,01765		0,01525+0,035 (2) 0,02513	0,0160+0,0171 (2) 0,01655	0,0115—0,028 (2) 0,0197	0,0208—0,028 (5) 0,02692	0,0161+0,034 (2) 0,02505
ATP	1,81—2,16 (4) 2,0	1,45—1,84 (6) 1,68	1,14—1,47 (6) 1,29	0,62—0,87 (4) 0,70	0,515	0,41—0,78 (4) 0,55	0,035—0,052 (3) 0,045	0,99—1,05 (3) 1,022	0,22—0,42 (5) 0,31	0,041—0,19 (4) 0,099
ADP	0,21—0,31 (4) 0,28	0,42—0,67 (5) 0,57	0,62—0,97 (5) 0,839	0,71—0,87 (3) 0,773		0,49+0,50 (2) 0,495	0,374+0,53 (2) 0,444	0,88+0,89 (2) 0,885	0,556—0,824 (5) 0,657	0,45—0,536 (3) 0,495
ATP/ ADP	6,2—8,3 (4) 7,3	2,44—3,62 (5) 2,99	1,19—1,84 (5) 1,58	0,575+0,932 (2) 0,844		0,98+1,11 (2) 1,045	0,066+0,139 (2) 0,103	1,16+1,20 (2) 1,18	0,370—0,622 (5) 0,489	0,09—0,38 (3) 0,21

TAB. 2.—METABOLITKONZENTRATIONEN IM GEHIRN VON NORMAL- UND NULLTIEREN, NACH ISCHÄMIE- UND ANOXIEVERSUCHEN BEI 26°C KÖRPERTEMPERATUR

Alle Angaben in 10^{-3} mol/l.

			Ischämie 26°c						Anoxie 26°c		
	Normaltiere	Nulltiere	1 min J.	1,5 min J.	2 min J.	5 min J.	10 min J.	25 min J.	10 min A.	15 min A.	25 min A.
Milch-säure	1 42 – 2,75 (5) 2,19	1 59 – 3,93 (8) 2,35	2,41 – 4,84 (5) 3,25	2,13 – 5,88 (5) 3,96	4,0 – 5,07 (3) 4,6	8,23	10,2 – 12,7 (5) 10,81	10,5 – 20,0 (3) 16,6	11,7 – 13,6 (3) 12,6	16,3 – 17,1 (3) 16,8	20,4 – 22,3 (3) 21,6
anorg. P	1,73 – 3,03 (7) 2,44	2,65 – 4,0 (10) 3,19	4,13 – 5,0 (5) 4,57	4,16 – 5,05 (6) 4,61	4,0 – 5,0 (4) 4,51	5,8	5,07 – 7,6 (6) 6,22	10,1 – 11,2 (3) 10,5	6,05 – 8,7 (3) 7,15	7,75 – 9,2 (3) 8,53	7,3 – 13,2 (4) 10,2
PKr	2,87 – 3,8 (7) 3,51	2,06 – 3,72 (10) 2,94	1,2 – 2,57 (5) 1,85	1,14 – 2,3 (6) 1,44	1,64 – 2,05 (4) 1,83	1,4	0,61 – 1,74 (6) 0,99	(3) 0	0,515 – 1,13 (3) 0,785	(3) 0	(3) 0
FDP	0.019 – 0.034 (4) 0,026	0,030 – 0.051 (4) 0.0414	0,088 – 0,1 (3) 0,095	0.065 – 0,093 (3) 0,0835	0,067 – 0,105 (3) 0,088		0.067 – 0.076 (4) 0.071	0,041 – 0,087 (3) 0,0654	0,063 + 0,070 (2) 0,0665	0,052 + 0,066 (2) 0,059	0,032 – 0,085 (4) 0,053
BTS	0.008 – 0,0192 (4) 0,0151	0,0126 – 0,0219 (4) 0,01611	0,01335 + 0,020 (2) 0,01678	0,01375 – 0,0268 (3) 0,01952	0,0115 – 0,015 (3) 0,01338		0,0104 – 0,0237 (4) 0,01676	0,0092 – 0,023 (3) 0,0146	0,015 + 0,0522 (2) 0,0336	0,0080 + 0,0145 (2) 0,01125	0,007 – 0,013 (3) 0,01037
DAP	0,0048 – 0,0204 (4) 0,0141	0,0152 – 0,030 (4) 0,02175	0,0216 + 0,0283 (2) 0,02495	0,0217 – 0,0275 (3) 0,0254	0,024 – 0,0285 (3) 0,02553		0,0143 – 0,0242 (4) 0,0203	0,0104 – 0,0242 (3) 0,01806	0,0157 + 0,022 (2) 0,01885	0,016 + 0,017 (2) 0,0165	0,0104 – 0,027 (3) 0,0169
ATP	1.95 – 2,27 (6) 2,06	1,7 – 2,26 (4) 1,955	1,59 – 2,1 (5) 1,85	1,7 – 2,02 (3) 1,86	1,65 – 1,83 (4) 1,74	1,4	1,05 – 1,34 (3) 1,23	0,29 – 0,237 (3) 0,27	1,15 + 1,47 (2) 1,31	0,63 – 1,02 (3) 0,85	0,235 – 0,29 (3) 0,26
ADP	0,249 – 0,35 (4) 0,31	0,320 – 0,476 (4) 0,391	0,55 – 0,72 (3) 0,64	0,531 – 0,652 (3) 0,608	0,535 – 0,7 (3) 0,636		0,67 – 0,85 (4) 0,785	0,565 – 0,68 (3) 0,61	0,865 + 0,666 (2) 0,765	0,677 + 0,773 (2) 0,725	0,50 – 0,64 (4) 0,569
ATP/ ADP	5,94 – 8,03 (4) 6,605	3,8 – 7,05 (4) 5,165	2,33 – 2,89 (3) 2,69	2,66 + 2,84 (2) 2,75	2,45 – 3,42 (3) 2,8		1,67 + 1,96 (2) 1,82	0,42 – 0,47 (3) 0,438	1,7 + 1,73 (2) 1,715	0,935 + 1,14 (2) 1,038	0,37 – 0,49 (3) 0,445

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Phosphokreatin entspricht (THORN *et al.*, 1955). Beim Normaltier (Abb. 1 u. 2) iegen die niedrigsten Werte dieser Fraktion um 2,9 mmol/l., beim Nulltier dagegen bei rund 2,0 mmol/l., während man unter günstigen Bedingungen in beiden Reihen bis zu 3,8 mmol/l. findet (LINDBERG u. ERNSTER, 1950: 3,26 mmol/l.). Es bleibt eine mittlere Differenz von 0,6–0,8 mmol/l. zwischen Normal- und Nulltieren bestehen. Die höheren Werte bei den Nulltieren gegenüber unseren früheren Versuchen dürften durch die inzwischen gesammelte experimentelle Erfahrung und durch unsere verbesserte Versuchstechnik bedigt sein.

TAB. 3.—KONZENTRATIONEN AN AMINOSÄUREN IM KANINCHENGEHIRN
Alle Angaben in 10^{-3} mol/l.

			Ischämie 37°C			
	Normaltiere	Nulltiere	2 min J.	5 min J.	9,15 min J.	10 min J.
Alanin		0,928+1,04 (2) 0,984	0,876+0,894 (2) 0,885	1,27	1,11	1,29
Asparagin-säure	1,95–2,24 (4) 2,09	2,12–2,28 (3) 2,19	2,15–2,25 (3) 2,2	2,1+2,17 (2) 2,14	2,18	3,58
Glutamin-säure	8,65–10,49 (3) 9,29	11,23+11,35 (2) 11,29	8,23+9,63 (2) 8,93	9,19	10,13	

			Ischämie 26°C				
	Normaltiere	Nulltiere	1 min J.	1,5 min J.	2 min J.	5 min J.	10 min J.
Alanin	1,169	0,472	1,169+1,235 (2) 2,202	1,158		1,028	1,09
Asparagin-säure	2,08+2,45 (2) 2,27	1,9–3,43 (4) 2,58	1,8–2,2 (4) 2,02	1,88–2,15 (3) 2,04	1,83+2,32 (2) 2,08	1,76	1,7–2,15 (3) 1,98
Glutamin-säure	7,37–9,6 (3) 8,16		9,06+9,53 (2) 9,3	8,04–8,08 (3) 8,07		10,55	8,33

Zusammenfassende Arbeiten WAELSCH (1953), WEIL-MALHERBE (1953).

Die Konzentration an ATP (Abb. 3 u. 4) fällt bei 37°C zwischen Normaltier und Nulltier um 0,35 mmol/l. im Mittel ab, bei 26°C hingegen nur um 0,1 mmol. Das ADP steigt bei 37°C um 0,29 mmol und bei 26°C um 0,08 mmol im Mittel an.

Die Differenzen in der Konzentration an ATP und ADP zwischen beiden Versuchsreihen erscheinen auf den ersten Blick gering. Eine Betrachtung des

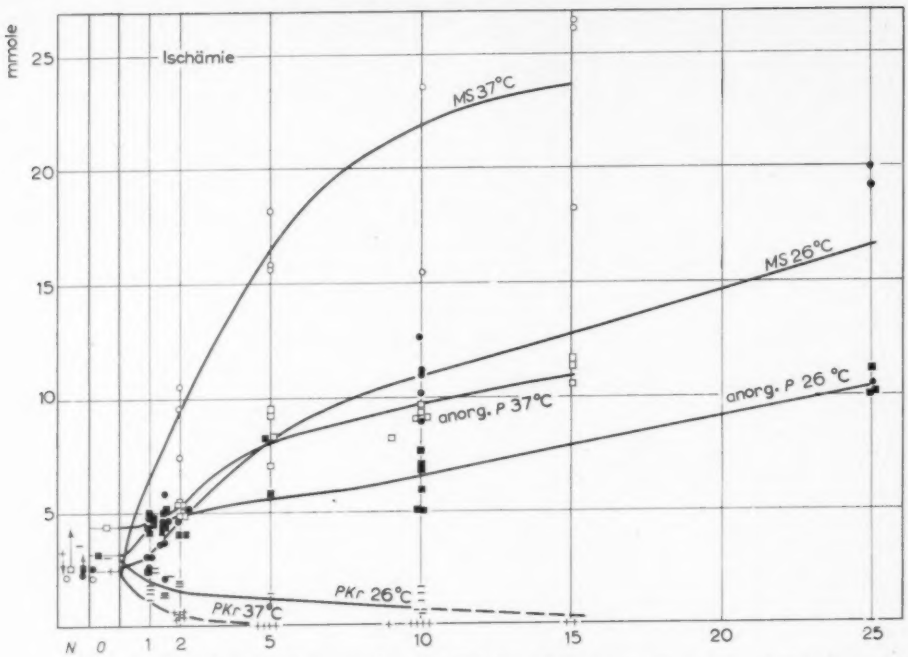

ABB. 1.—Verhalten der Konzentration an Milchsäure (MS), anorg. Phosphat und Phosphokreatin (PKr) in der Ischämie bei 37°C und 26°C Körpertemperatur. Wegen der besseren Übersicht in Spalte 1 (N = Normaltiere) und Spalte 2 (O = Nulltiere) nur Wiedergabe der Mittelwerte. Die Pfeile zeigen den Konzentrationsunterschied von anorg. Phosphat und PKr zwischen Normal- und Nulltieren an. Alle Konzentrationsangaben in mmol/l. Eingezeichnete Linien sind Sichthilfen.

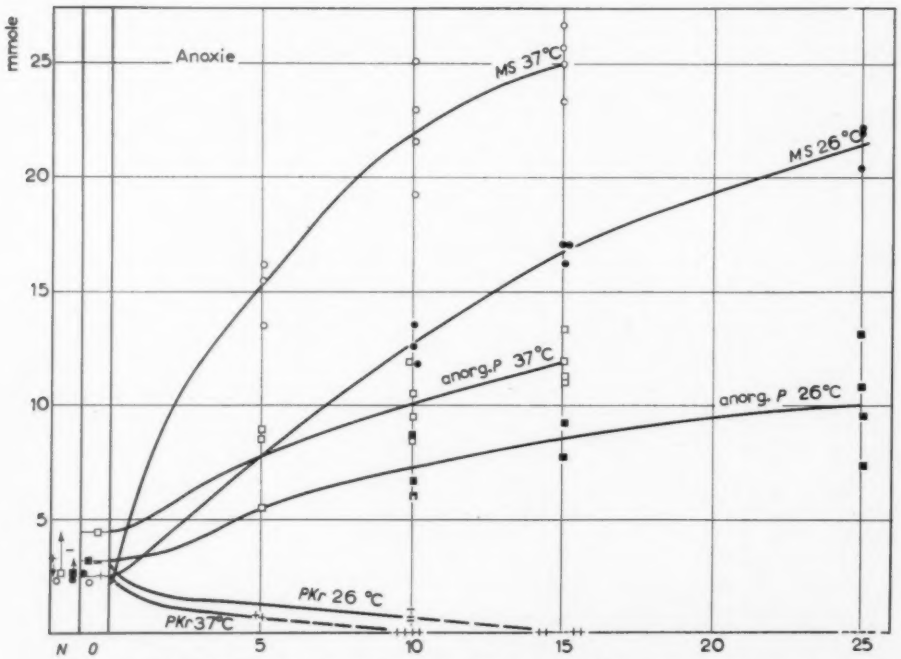

ABB. 2.—Veränderung der Konzentration an MS, anorg. Phosphat und PKr unter N_2-Beatmung (Anoxie) bei 37°C und 26°C. N = Mittelwerte der Normaltiere; O = Mittelwerte der Nulltiere. Pfeile zur Demonstration der Konzentrationsunterschiede zwischen Normal- und Nulltier. Eingezeichnete Linien sind Sichthilfen.

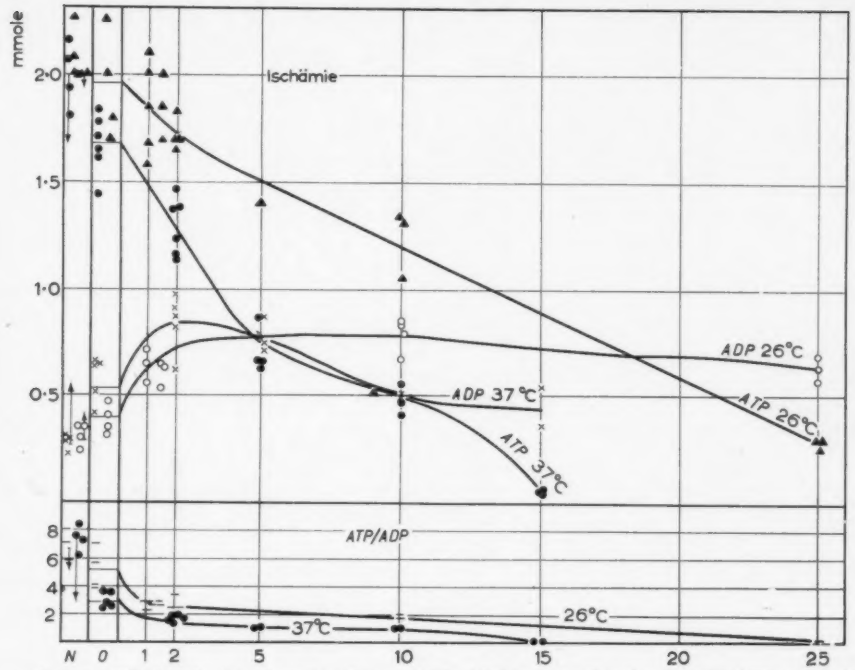

Abb. 3.—Wiedergabe der Konzentration an ATP, ADP und der Quotienten ATP/ADP am Normaltier (N), Nulltier (O) und in der Ischämie bei 37°c und 26°c Körpertemperatur. Eingezeichnete Linien sind Sichthilfen.

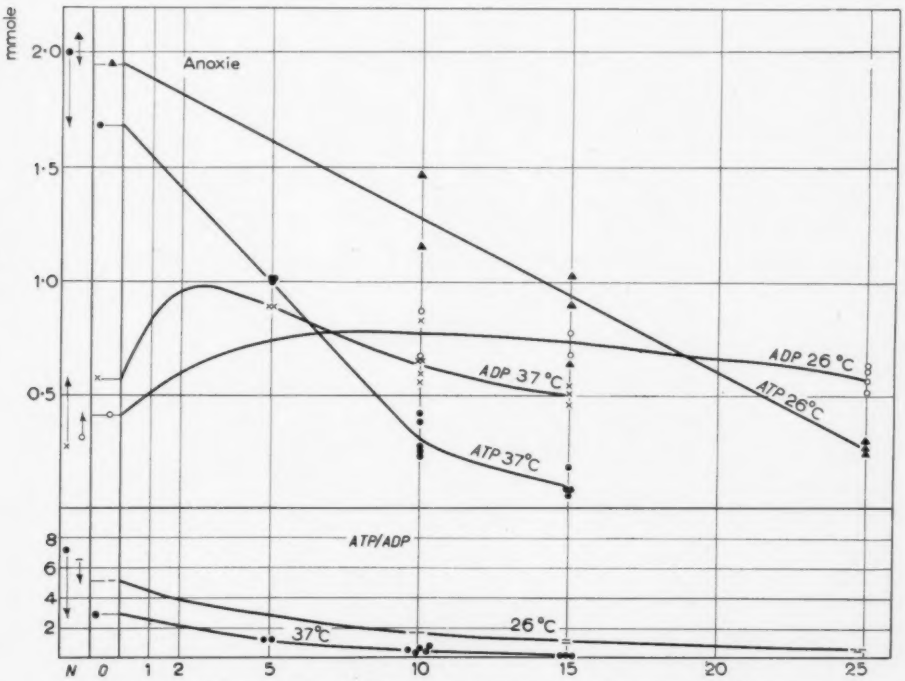

Abb. 4.—Verhalten von ATP, ADP und dem Quotienten ATP/ADP unter N_2-Beatmung (Anoxie) bei 37°c und 26°c Körpertemperatur. Eingezeichnete Linien sind Sichthilfen.

Quotienten ATP/ADP zeigt jedoch bei 37°C einen mittleren Abfall von 7,30 auf 2,99, bei 26°C sinkt der Wert nur von 6,61 auf 5,17.

Für des FDP (Abb. 5a u. b) konnten wir die früher beschriebene Zunahme um das Dreifache am Nulltier gegenüber dem Normaltier bestätigen (THORN *et al.*, 1955). Bei 26°C steigt der Gehalt von 0,026 mmol/l. auf 0,0414 mmol/l., also um 60 Prozent an.

Die MS-Konzentration zeigt keine Veränderung, für die Metabolite DAP und BTS lässt sich keine Relation finden (Tab. 1 u. 3). Ueber die Änderungen an freiem NH_3 wird an anderer Stelle berichtet (THORN u. HEIMANN, 1957*a*).

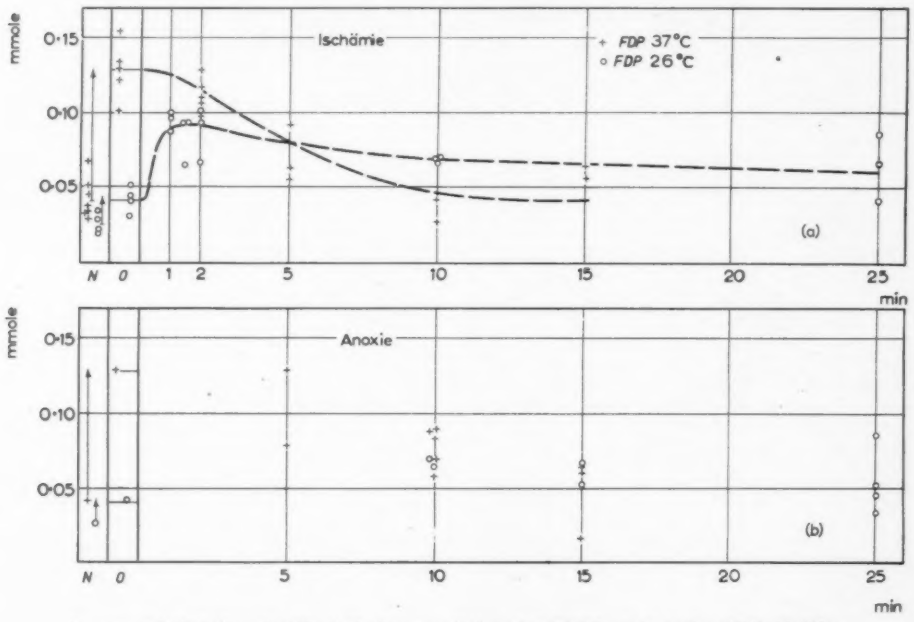

ABB. 5.—(a) Verhalten der Werte für Fructosediphosphat (FDP) am Norntier al(N), Nulltier (O) und in der Ischämie bei 37°C und 26°C Körpertemperatur. Eingeszeichnete Linien sind Sichthilfen.
(b) Angaben über den Gehalt an FDP in der Anoxie bei 37°C und 26°C Körpertemperatur.

Ischämie- und Anoxieversuche (Abb. 1–5, Tab. 1–3)

Aus den Abb. 1–5 geht hervor, dass praktisch kein Unterschied in den Konzentrationen der untersuchten Metabolite zwischen Ischämie und Anoxie besteht. Die für die einzelnen Metabolite typischen Veränderungen treten in beiden Versuchsreihen etwa nach gleichen Versuchszeiten auf, wenn man berücksichtigt, dass die Anoxie nicht so akut einsetzt wie die Ischämie. Diese Feststellung gilt auch für beide Versuchsreihen bei 26°C Körpertemperatur.

Der Gehalt an Phosphokreatin (PKr), welcher schon beim Nulltier rund 0,5–0,6 mmol/l. niedriger gefunden wird als beim Normaltier, sinkt in der Ischämie bei 37°C sehr rasch ab. Nach 2 min finden wir noch rund 0,6 mmol/l.; nach 5 min gelingt der Nachweis nicht mehr. In der Anoxie bei 37°C wird nach 5 min noch in

zwei Fällen ein Gehalt von 0,7 mmol/l. gefunden, nach 9 und 10 min glückt auch hier der Nachweis nicht mehr. Bei 26°C verläuft der Abfall des PKr flacher. Nach 2 min Ischämie findet man bis zu 2 mmol/l. PKr, in allen untersuchten Fällen nach 10 min 0,6–1,7 mit einem Mittel von 0,99 mmol/l. Die Werte bei 10 min Anoxie liegen zwischen 0,51 und 1,13 mmol/l.

Der ATP-Gehalt nimmt während der Anoxie und Ischämie ab. Für die Versuchstemperatur von 26°C kann man den bis 25 min beobachteten, langsamen Abfall der Konzentration etwa als linear bezeichnen (Abb. 3 u. 4). Bei 37°C kommt es nach einer Verminderung des ATP-Gehalts auf 0,5–1,6 mmol/l., der nach 5–10 min Versuchsdauer erreicht wird, zu einer Verlangsamung des Konzentrationsabfalls. Diese Beobachtung stimmt mit unseren früheren Versuchen überein. Hervorgehoben sei der noch relativ hohe Gehalt an ATP nach 10 min Anoxie (0,22–0,42 mmol/l. oder Ischämie (0,41–0,78 mmol/l.). Erst nach einer Versuchszeit von 15 min liegen die Werte unter 0,1 mmol/l. Der Gehalt an ADP steigt bei 37°C und 26°C in Anoxie und Ischämie an. Bei normaler Körpertemperatur sinkt die Konzentration nach 10 min Belastung wieder auf die am Nulltier gefundenen Werte ab. Bei 26°C liegt die Konzentration nach 25 min höher als die der Ausgangswerte.

Das Verhältnis ATP/ADP fällt bei 37°C nach 4–5 min auf den Wert 1 ab, bei 26°C wird diese Relation etwa nach 15 min erreicht.

Das anorg. Phosphat (Abb. 1 u. 2) steigt bei 37°C in Anoxie und Ischämie von rund 4 mmol/l. auf 11–12 mmol/l. nach 15 min an. Die grösste Zunahme tritt in den ersten Minuten nach Versuchsbeginn ein. Bei 26°C erfolgt die Phosphatfreisetzung gleichmässig. Der Gehalt steigt in 25 min von rund 3 auf 10 mmol/l.

Das Fructosediphosphat (FDP) (Abb. 5a u. b) steigt bei 37°C im Gehirn der Nulltiere gegenüber dem der Normaltiere um das Dreifache. Wir finden am Nulltier einen Mittelwert von 0,128 mmol/l. Während der Anoxie und Ischämie fällt die Konzentration langsam ab und erreicht in beiden Versuchsreihen nach 15 min etwa 0,05 mmol/1. Beim Vergleich mit unseren früheren Werten (THORN *et al.*, 1955) sei die wesentlich geringere Streuung der Konzentration dieses Metaboliten hervorgehoben. Während wir bei 37°C keinen Anstieg der FDP-Konzentration gegenüber dem Nulltier finden, lässt sich eine Zunahme bei 26°C nachweisen. In den ersten Minuten steigt der Gehalt an FDP von 0,64 auf 0,1 mmol/l. an.

Im Rahmen der durchgeführten Untersuchungen finden wir am MS-Gehalt des Gehirns die grössten Veränderungen. Von rund 2 mmol/l. am Normal- und Nulltier steigt die Konzentration bei 37°C in 15 min auf 25–26 mmol/l. Die stärkste MS-Bildung liegt in den ersten Minuten, sie beträgt nach 2 min 7 mmol/l. Bei herabgesetzter Körpertemperatur erfolgt die MS-Bildung entsprechend langsamer. Nach 25 min ist der Gehalt von 2,05 mmol/l. auf etwa 20 mmol/l. angestiegen. Bemerkenswert ist die Tatsache, dass bei dieser Temperatur der steile Anstieg im Beginn der Belastung ausbleibt. Der mittlere Gehalt hat nach 1 min von 2,35 auf 3,25 (0,9) mmol/l., nach 1½ min auf rund 4,0(1,65) und nach 2 min auf 4,6 (2,25) mmol/l. zugenommen. Die geringe Änderung von 0,9 mmol/l. in der ersten Minute entspricht den Befunden von THORN u. RACZKOWSKI (1952) am dekapitierten Tier. Hier setzte eine messbare Zunahme des MS-Gehalts bei 26°C erst nach 40 sec ein.

Von verschiedenen Gehirnen haben wir einen Teil abgezweigt und daraus Kochsäfte hergestellt, die zur Bestimmung von Alanin, Asparaginsäure und Glutaminsäure

dienten. Wir wollten prüfen, ob sich die Konzentrationen dieser Aminosäuren ändern, von denen erwiesen ist, dass sie in Zwischenstufen des Kohlenhydratabbaus oder des Zitronensäure-Cyclus überführt werden können. Die ermittelten Werte sind in Tab. 3 wiedergegeben. Soweit die Werte eine Beurteilung zulassen, tritt im untersuchten Bereich keine von der Ischämie abhängige Veränderung ein. Die Konzentration an Glutaminsäure liegt zwischen 8–11 mmol/l., an Asparaginsäure um 2 mmol/l. und an Alanin um 1,0 mmol/l. Hervorgehoben sei, dass die beiden hohen Asparaginsäurewerte (3,43 und 3,58 mmol/l.) von zwei schwangeren Tieren stammen, die durch ein Versehen in Versuch genommen wurden (AWAPARA *et al.*, 1950: 3,65 mmol/l. Asparaginsäure, Gehirn, Ratte; DAWSON, 1950: 10,7 mmol/l. Glutaminsäure, Gehirn, Ratte; KREBS *et al.*, 1949: 9,93 mmol/l. Glutaminsäure, Gehirn, Katze).

Für die hier referierten Werte an BTS und DAP lässt sich keine Beziehung zur ischämischen oder anoxischen Belastung finden (THORN *et al.*, 1955).

DISKUSSION

Unsere Bemühungen sind darauf gerichtet, am Versuchsmodell der Kreislaufunterbrechung (Ischämie), des O_2-Mangels (Anoxie) und der Erstickung (Asphyxie) zu prüfen, in welcher Weise einzelne Organe während der Noxe auf die gestörte Energieversorgung reagieren, und wie sich der Kreislauf verhält. Um diesem Ziel ein Stück näher zu kommen, haben wir an allen Tieren EEG, EKG und Blutdruck fortlaufend registriert, damit ein Vergleich der Tierversuche untereinander möglich ist, und damit man den Weg verfolgen kann, der zu den Veränderungen im Stoffwechsel geführt hat. Besonders wichtig erschien uns die fortlaufende Erfassung des Blutdrucks. Zu diesem Zweck konstruierten wir ein elektrisches Messgerät von hoher Empfindlichkeit, über das SCHOLL u. THORN (1957) berichten. Neben dem Gehirn wurden Leber, Herz, Niere und Muskulatur desselben Tieres in die Untersuchungen einbezogen und die oben aufgezählten Metabolite bestimmt. Die Durchführung der zahlreichen Bestimmungen neben den Tierversuchen in relativ kurzer Zeit war nur möglich, weil die beschriebenen optischen Fermentteste nicht nur einen hohen Gewinn an Spezifität bei der Bestimmung aus dem Gewebsextrakt darstellen, sondern auch eine erhebliche Zeitersparnis gegenüber analytisch-chemischen Methoden bedeuten.

Man darf auf Grund der vorliegenden Befunde vielleicht folgende nicht nur für das Gehirn geltende Bemerkung über den Zusammenhang zwischen Energieversorgung, Durchblutung und Funktion machen:

Ein gut versorgtes Organ enthält einen für den jeweiligen Metaboliten charakteristischen niedrigen Gehalt an anorg. Phosphat, ADP, FDP, DAP, BTS, MS und einen entsprechend hohen Wert an Glukose, Glycogen, ATP und PKr. (Beim Phosphokreatin machen Leber und Niere offenbar eine Ausnahme (THORN *et al.*, 1957*b*). Der Quotient ATP/ADP zeigt einen hohen Wert. Je nach spezifischer Stoffwechselaktivität des Organs und Art der Stoffwechselstörung werden diese Relationen gegeneinander verschoben.

Entsprechend der obigen Anmerkung darf man für das Gehirn unter ischämischer und anoxischer Belastung als Mass für die Stoffwechselaktivität auch nach dem Verschwinden der elektrischen Spontanpotentiale die MS-Bildung werten und in Abhängigkeit von der anaeroben Glykolyse die übrigen Metabolite betrachten.

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Zur Frage der Hypothermie ist es sicher ein wichtiger Befund, dass man für den mittleren MS-Gehalt bei 26°C und 37°C im Gehirn und in allen anderen untersuchten Organen desselben Kaninchens etwa den gleichen Wert, keinesfalls einen wesentlich höheren, findet. Von WINTERSTEIN (1954) wird die Annahme vertreten, dass es bei Unterkühlung zu einer Störung der O_2-Versorgung und damit zu einer Ansäuerung des Gewebes kommt, die letztlich den Tod bedinge. Für die Entstehung der Acidose kommt jedoch nur eine MS-Anhäufung in Frage. Eine andere organische Säure, die eine derartige Konzentrationsänderung erfährt, ist uns unbekannt. Eine solche MS-Änderung ist nur möglich bei einer schweren Störung der Atmung oder der Herzfunktion. Bis zu einer Temperatursenkung von 26°C kann man diese Acidosetheorie für Gehirn, Herz, Leber, Niere und Muskulatur ausschliessen.

Der steile MS-Anstieg bei 37°C in 10 min auf eine mittlere Konzentration von 22–23 mmol/l. oder auf rund 25 mmol/l. nach 15 min wird erst durch die in diesen Versuchsreihen gewählte längere Versuchszeit von 15 und 25 min deutlich. Die hohen Werte werden bilanzmässig verständlich, wenn man berücksichtigt, dass in der Urethannarkose der Gehalt an freier Glucose im Blut ansteigt (ERÖZ *et al.*, 1951; KUN u. ABOOD, 1949). Unsere noch nicht publizierten Werte (THORN *et al.*, 1957*c*) stimmen mit denen von KERR (1937) und KUN u. ABOOD (1949) überein. Freie Glucose und Glycogen des Gehirns entsprechen offenbar summarisch der Glucose des Blutes (KERR, 1937). Der hohe MS-Anstieg in 10–15 min dürfte wesentlich für die von THORN und HEITMANN (1954) unter den gleichen ischämischen und anoxischen Bedingungen gemessenen Änderungen des pH Wertes an der freien Gehirnoberfläche sein (Abfall des pH Wertes von 7,24 auf 6,4–6,3). Die bei THORN u. RACZKOWSKI (1952) diskutierte Abnahme der MS-Bildungsgeschwindigkeit etwa 2–3 min nach der Decapitation trifft nach unseren heutigen Befunden am intakten Gehirn nicht zu. In der Ischämie dürfte etwa bis zur 5. Minute eine lineare Änderung gesichert sein, später streuen die Werte, so wie auch die Ausgangswerte für Glycogen und Glucose schwanken.

Der MS-Anstieg in der Anoxie erfolgt gleichmässiger als in der Ischämie, weil der Kreislauf noch für 3–4 min intakt ist und während dieser Zeit Glucose nachliefern kann. Während der Ischämie kann nur umgesetzt werden, was sich im Moment der Kreislaufunterbrechung im Gehirn an Glucose befindet. Die geringe Änderung des MS-Gehalts bei 26°C um 0,9 mmol/l. nach 1 min Kreislaufunterbrechung dürfte eine Bestätigung früherer Befunde sein, dass die Glycolyse bei 26°C erst nach einer Latenzzeit von etwa 40 sec einsetzt (THORN u. RACZKOWSKI, 1952).

Die eigentliche Bedeutung der MS-Bildung liegt darin, einerseits für Phosphorylierungsprozesse erforderliches ATP zu synthetisieren, andererseits durch die Abgabe von Wasserstoff an BTS oxydiertes DPN für weitere Substratdehydrierungen zu gewinnen, Reaktionen, die wir mit unseren kristallisierten Fermentpräparaten für die Bestimmung von ATP, ADP und BTS nachahmen. Der bilanzmässige Gewinn der anaeroben Glycolyse beträgt bei einem Endwert von rund 25 mmol/l. und einem Anfangswert von rund 2 mmol/l. MS im Mittel etwa 23 mmol/l. an ATP.

Ebenso stehen rund 23 mmol/l. DPN ox für weitere Dehydrierungen zur Verfügung. Der gesamte Umsatz an ATP in der Versuchszeit beträgt rund 48 mmol/l., da am Beginn des Versuchs etwa 2 mmol/l. vorliegen, die am Ende ebenfalls verbraucht sind. Weiter kommen 3–4 mmol/l. PKr hinzu, die wohl ebenfalls zur ATP-Synthese dienen. Man darf die jeweils ermittelten Werte für ATP, ADP, FDP,

anorg. Phosphat, etc. nicht als unabhängige Grössen sondern als Glieder eines Fliessgleichgewichts betrachten, das unter anaeroben Bedingungen durch den Abbau von Glucose und Glycogen zu MS aufrecht erhalten wird. Das mit dem Tod endende Experiment beweist aber, dass die anaerob freigesetzte Energie nicht ausreicht. Gleichzeitig erhebt sich die Frage der Energieausnutzung, die wir nicht beantworten können; denn in der Ischämie und nach Aufhören des Kreislaufs auch in der Anoxie bilden sich im Gehirn erhebliche pH-Verschiebungen zum sauren Bereich aus (THORN u. HEITMANN, 1954).

Den Gehalt an PKr betrachten wir als ein schnell verfügbares Energiereservoir, dessen wahre Konzentration bei normaler Körpertemperatur wegen der Spaltung durch den Reiz der Gewebsentnahme nur schwer gemessen werden kann. Für die Ermittlung der Konzentration dieses Metaboliten wirkt sich eine Senkung der Körpertemperatur besonders günstig aus.

Den Anstieg des Gehalts an FDP gegenüber dem Normaltier können wir hier nur registrieren, über die Ursache wissen wir nichts.

Aus den beschriebenen Aminosäurebestimmungen kann man nicht ersehen, ob in der Ischämie und Anoxie Änderungen in der Konzentration auftreten. Zur Klärung dieser Frage müssen weitere Untersuchungen unternommen werden. Die bisher ermittelten Schwankungen in der Konzentration der einzelnen Aminosäuren werden von anderen Faktoren als von der Versuchsdauer abhängen. Wir verfolgten hier zunächst das Ziel, die Möglichkeit der Anwendung von optischen Fermenttesten auch auf diesem Sektor zu prüfen; denn es gelingt eine quantitative Aussage im Gegensatz zu anderen Bestimmungsmöglichkeiten in wenigen Minuten. Wir möchten nicht annehmen, dass man bei den von Tier zu Tier auftretenden Konzentrationsschwankungen (z.B. der Glutaminsäure) eine Deutung der Befunde im Sinne von VRBA (1956) machen kann.

ZUSAMMENFASSUNG

An 170 trepanierten Kaninchen von normaler und herabgesetzter Körpertemperatur (26°C) wurden Ischämieversuche mit Hilfe einer Halsmanschette (OPITZ u. THORN, 1949; THORN *et al.*, 1955) und Anoxieversuche durch Beatmung mit $N_2 + CO_2$ durchgeführt. Die mit einem vorgekühlten Löffel rasch entnommenen und in flüssiger Luft eingefrorenen Gehirne dienten zur Bestimmung von ATP, ADP, Fructosediphosphat (FDP), Dioxyacetonphosphat (DAP), Brenztraubensäure (BTS), Milchsäure (MS), anorg. Phosphat, Alanin, Glutaminsäure und Asparaginsäure unter Anwendung optischer Fermentteste (THORN *et al.*, 1955; PFLEIDERER *et al.*, 1955*a*, *b*) Durch den Schock des Decerebrierens werden die Konzentrationen an anorg. Phosphat, PKr, FDP, ATP, ADP momentan verändert. Die Werte für ADP, FDP und anorg. Phosphat steigen an, für PKr und ATP fallen sie ab. Durch Einfrieren des ganzen Kopfes mit dem Gehirn *in situ* bei einer Reihe von Tieren kann man die Grössenordnung dieser rasch eintretenden Änderung abgrenzen.

Die Art der Veränderungen ist in Anoxie und Ischämie weitgehend gleich. Das anorg. Phosphat steigt in der Ischämie und Anoxie bei 37°C in 15 min von etwa 4 mmol/l. auf rund 10 mmol/l., bei 26°C von etwa 3 auf 10 mmol/l. in 25 min.

Die MS-Konzentration wächst von etwa 2 mmol/l. auf rund 25 mmol/l. nach 15 min bei 37°C und auf etwa 20 mmol/l. in 25 min bei 26°C. Der Gehalt an PKr fällt rasch ab, und es ist bei normaler Körpertemperatur nach einigen Minuten

nicht mehr nachzuweisen, bei 26°C gelingt der Nachweis in allen untersuchten Fällen noch nach 10 min.

ATP wird zu rund 2 mmol/l. im Gehirn gefunden, trotz Neubildung sinkt der Gehalt langsam ab, es ist aber nach 10–15 min bei 37°C und nach 25 min bei 26°C in allen Fällen nachweisbar. Der Wert für ADP steigt in den ersten Minuten der Anoxie und Ischämie von 0,3–0,5 mmol/l. bis zu 1,0 mmol/l. und fällt dann später ab. Am *in situ* eingefrorenen Gehirn werden für den Quotienten ATP/ADP Werte bis zu 8 gefunden; die Werte fallen zunächst rasch ab, erreichen nach 4–5 min bei 37°C den Wert 1, bei 26°C nach etwa 15 min, später bleibt die Konzentration an ATP stets unter der des ADP.

Herrn cand. med. F. LIEMANN danken wir für umsichtige Mithilfe bei den Tierversuchen.

SUMMARY

Rabbits (170) with trepanned skulls were studied under conditions of ischaemia and of anoxia. The experiments were performed at normal and at lowered (26° C) body temperatures. Ischaemia was induced by means of a cuff around the animals' neck (OPITZ and THORN, 1949; THORN *et al.*, 1955), and anoxia was obtained by ventilation with a mixture of N_2 and CO_2. The brains were taken out with a cooled spoon and frozen in liquid air. Deproteinized extracts served for the determination of ATP, ADP, fructosediphosphate (FDP), dihydroxyacetone phosphate (DAP), pyruvic acid (BTS), lactic acid (MS), inorganic phosphate, alanine, glutamic acid and aspartic acid, which were partly estimated by optical enzymic methods (THORN *et al.*, 1955; PFLEIDERER *et al.*, 1955*a* and *b*). The shock of the decerebration momentarily affected the concentrations of inorganic phosphate, creatine phosphate (PKr), FDP, ATP, and ADP. The concentration of inorganic phosphate was raised, and PKr and ATP lowered. The extent of this immediate change could be measured by comparison with the values obtained after freezing the whole head with the brain *in situ*.

The changes were of similar kind under anoxic and under ischaemic conditions. The inorganic phosphate increased in ischaemia and in anoxia from about 4 m-moles/l. to about 10 m-moles/l. within 15 min at 37°C; at a temperature of 26°C it increased from about 3 to 10 m-moles/l. within 25 min.

The concentration of lactic acid increased from about 2 m-moles/l. to about 25 m-moles/l. within 15 min at 37°C, and to about 20 m-moles/l. within 25 min at 26°C. The content of PKr decreased rapidly. It disappeared after a few minutes at normal body temperature. However, in all animals studied at 26°C it was still detectable after 10 minutes.

The brain ATP level was found to be about 2 m-moles/l. The content decreased gradually in spite of a new formation; but, it was still detectable in every case after 10 to 15 minutes at 37°C, and after 25 minutes at 26°C. In anoxia and in ischaemia the ADP-concentration initially increased from 0·3–0·5 m-moles/l. to 1·0 m-moles/l. and decreased later on.

The quotient ATP/ADP reached a value of 8 for brains frozen *in situ*. It decreased rapidly at the beginning. The quotient was 1 after 4–5 min at 37°C, and after 15 min at 26°C. Thereafter the concentration at ATP was constantly lower than that of ADP.

ANHANG

Für MS, anorg. P und PKr haben wir die Mittelwertsabweichungen in den einzelnen Messreihen gesondert berechnet und mit Hilfe der *t*-Verteilung die Unterschiede auf ihre Signifikanz geprüft.

		arith. Mittel	Standardabw.
MS	(1) Normaltiere 37°c	2,20	0,43
	(2) Nulltiere 37°c	2,15	0,37
	(3) Normaltiere 26°c	2,19	0,59
	(4) Nulltiere 26°c	2,35	0,70

		t	*F*
Vergleich der Mittelwerte:	(1) u. (2)	0,24	13
	(1) u. (3)	0,03	8
	(2) u. (4)	0,75	16
	(3) u. (4)	0,44	11

		arith. Mittel	Standardabw.
anorg. P	(1) Normaltiere 37°c	2,53	0,51
	(2) Nulltiere 37°c	4,37	0,55
	(3) Normaltiere 26°c	2,44	0,61
	(4) Nulltiere 26°c	3,19	0,37

		t	*F*	Sicherungsgrenze
Vergleich der Mittelwerte:	(1) u. (2)	6,53	13	0,1%
	(1) u. (3)	0,31	14	
	(2) u. (4)	4,66	14	0,1%
	(3) u. (4)	2,90	15	5%

		arith. Mittel	Standardabw.
PKr	(1) Normaltiere 37°c	3,26	0,30
	(2) Nulltiere 37°c	2,47	0,77
	(3) Normaltiere 26°c	3,51	0,28
	(4) Nulltiere 26°c	2,94	0,49

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		t	F	Sicherungsgrenze
Vergleich der Mittelwerte:	(1) u. (2)	2,38	13	5%
	(1) u. (3)	1,71	14	
	(2) u. (4)	0,13	14	
	(3) u. (4)	3,05	15	1%

Sehr signifikante Unterschiede bestehen zwischen den Mittelwerten des anorg. P für Normal—und Nulltiere bei 37°c Körpertemperatur, für Nulltiere von 37°c und Nulltiere von 26°c, ferner zwischen den Mittelwerten des PKr für Normal—und Nulltiere bei 26°c.

Signifikante Abweichungen ergeben sich zwischen dem anorg. P der Normal—und Nulltiere bei 26°c und für das PKr der Normal—und Nulltiere bei 37°c.

Keine statistische Sicherung ist für die Mittelwertsdifferenzen der MS zu erbringen.

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BEEINFLUSSUNG DER AMMONIAK-KONZENTRATION IN GEHIRN, HERZ, LEBER, NIERE UND MUSKULATUR DURCH ISCHÄMIE, ANOXIE, ASPHYXIE UND HYPOTHERMIE*†

THE EFFECTS OF ANOXIA, ISCHAEMIA, ASPHYXIA AND REDUCED TEMPERATURE ON THE AMMONIA LEVEL IN THE BRAIN AND OTHER ORGANS

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(*Received* 12 *May* 1957)

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EINLEITUNG

TASHIRO (1922) konnte im Froschnerven freies Ammoniak nachweisen und zeigen, dass nach Reizung des Nerven eine messbare Steigerung der Ammoniak-Konzentration eintrat. WINTERSTEIN u. HIRSCHBERG (1925) bestätigten die Beobachtungen von TASHIRO und untersuchten darüber hinaus die Verhältnisse am isolierten Rückenmark. Sie beschrieben Stoffwechselvorgänge, die unter Abspaltung von Ammoniak verlaufen, und zeigten deren Beeinflussung durch Narcotica und durch Reizung des Nervensystems.

Seitdem haben sich viele Untersucher mit der Bildung von Ammoniak im Gewebe beschäftigt (ANSELL u. RICHTER, 1954; BLUMSON, 1957; BÜLOW u. HOLMES, 1932; DAWSON, 1950; KERR, 1942; KREBS, 1935. 1948; KREBS *et al.*, 1948; KRITZMANN, 1947; MUNTZ, 1953; PARNAS, 1932, PARNAS u. LUTWAK-MANN, 1935; RICHTER u. DAWSON, 1948; RIEBELING, 1931, 1934; RÖSCH u. TECAMP, 1928; ROWSELL, 1954; SCHWARTZ u. DIBOLD, 1932; STANOJEVIC, 1938; VRBA, 1955; WAELSCH, 1953, 1955; WEIL-MALHERBE 1953; WEIL-MALHERBE u. GREEN, 1955).

Die in der Literatur beschriebenen Werte, insbesondere die älteren, zeigen sehr grosse Schwankungen (BÜLOW u. HOLMES, 1932; RIEBELING, 1931; SCHWARZ u. DIBOLD, 1932), deren Ursache einerseits in der Durchführung der Tierexperimente, andererseits in den Bestimmungsmethoden zu suchen sind. Eine exakte Erfassung des freien Ammoniaks gelingt nur, wenn man das Gewebe unmittelbar einfriert und Bestimmungsmethoden benutzt, die labile Aminogruppen, wie sie im Glutamin und Asparagin vorliegen, schonen.

METHODIK

Normaltier: Der ganze Kopf des Tieres mit dem freigelegten Gehirn *in situ* wird in flüssiger Luft eingefroren, anschliessend wird dekapitiert und das Gehirn aus dem gefrorenen Schädel herauspräpariert.

Nulltier: Versuchstiere, deren Organe ohne ischämische (Gehirn), asphyktische und anoxische Belastung entnommen und sofort in flüssiger Luft eingefroren werden (Gehirn 1-2 sec, die übrigen Organe 3-4 sec bis zum Einwurf).

* Kurze Mitteilung XX internat. Physiologen-Kongress Brüssel (1956).

† Für finanzielle u. apparative Unterstützung danken wir der deutschen Forschungsgemeinschaft, dem Ministerium für Wirtschaft und Verkehr, Düsseldorf, u. der Gesellschaft der Freunde der Universität.

Tierversuche und Gewebsaufarbeitung

Im Moment der Kreislaufunterbrechung mit Hilfe einer Halsmanschette wird auch die Lungenatmung blockiert (OPITZ u. THORN, 1949; THORN *et al.*, 1955). Es entsteht für das Gehirn eine Ischämie. Im übrigen Organismus bleibt aber die Blutzirkulation noch einige Minuten aufrechterhalten, die Kohlensäure wird nicht mehr abgegeben, der noch in der Lunge vorhandene Sauerstoff wird verbraucht, so dass sich der Zustand einer echten Erstickung (Asphyxie) entwickelt. Über methodische Einzelheiten und Metabolitveränderungen im Gehirn bei Ischämie und Stickstoffbeatmung (Anoxie) berichten THORN *et al.* (1957*a*). Metabolitveränderungen während asphyktischer und anoxischer Belastung an Leber, Niere, Herz und Muskulatur sind von THORN *et al.* (1957*b*) beschrieben.

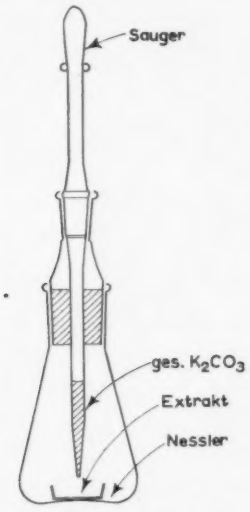

ABB. 1.—Reaktionsgefäss.

Alle Tierversuche sind ausschliesslich in Urethannarkose durchgeführt worden (1,0–1,2 g/kg), um eine unterschiedliche Beeinflussung des NH_3-Gehalts durch die Narkose weitgehend zu vermeiden (RICHTER u. DAWSON, 1948). Im Moment des Decerebrierens wird der Rumpf des Tieres weit eröffnet, die Organe in der Reihenfolge Leber, Herz, Niere herausgelöst und in flüssige Luft geworfen. Die Muskulatur entstammt jeweils dem linken Muskulus Quadriceps. Keines der Organe, über die wir hier berichten, war vor dem Einwerfen in die flüssige Luft länger als 3–4 sec von der Durchblutung und seiner Lage *in situ* getrennt. Auf diese Weise ist die Möglichkeit eines Vergleichs der einzelnen Gewebe nach einer bestimmten Belastung möglich.

Die Organe werden im Starmix unter Kohlensäureschnee pulverisiert und mit 6 ml M/3 Perchlorsäure pro Gramm Gewebe 15 min im Kühlraum extrahiert und 10 min hochtourig zentrifugiert (THORN *et al.*, 1955). Vom sauren Überstand pipettieren wir für die NH_3-Bestimmung einige ml ab, die sofort mit Kohlensäureschnee eingefroren und später in einer Tiefkühltruhe bei $-25°C$ aufbewahrt werden. Diese Bedingungen muss man streng einhalten. Ein verzögertes Einfrieren der Organe (etwa nach einer $\frac{1}{2}$ oder 1 min) führt zu einer erhöhten enzymatischen Desaminierung im Gewebe (RICHTER u. DAWSON, 1948). Auch das Aufbewahren der sauren Extrakte im Kühlraum oder mehrfaches Auftauen ergeben eine Ammoniak-Abspaltung aus labilen Aminogruppen durch das saure Enteiweissungsmittel.

NH_3-*Bestimmung*

NH_3-Bestimmung: (Abb. 1) $d = 2$ cm, $V = 4$ ml, Hg 436 mμ Photometer Eppendorf. $\Delta E/\mu$g Ammoniak—N = 0,06.

Prinzip: Der in 0,5 ml Gewebsextrakt enthaltene freie Ammoniak wird mit 0,5 ml gesättigter

K_2CO_3 Lösung ausgetrieben (Endkonzentration: Halbsättigung) und diffundiert direkt in 4 ml Nesslers Reagenz (Merck 9011 und 9012, 1 : 1 gemischt, 1 : 50 verdünnt).

Unsere Bestimmungsmethode sollte bei ständiger Verwendung von 0,5 ml Extrakt und 0,5 ml gesättigter K_2CO_3 Lösung empfindlich genug sein, um z.B. 0,3 von 0,2 μg NH_3 unterscheiden zu können, da wir mit so geringen Mengen an Ammoniak rechnen mussten. Grössere Extraktvolumina zu verwenden ist wegen einer Behinderung der Diffusion nicht ratsam. Die Diffusionszeit wiederum ist durch die Haltbarkeit von Nesslers Reagenz begrenzt. Für die Erfassung so geringer Mengen an NH_3 konnte nur das von CONWAY (1939) angegebene Prinzip der Diffusion im geschlossenen System in Frage kommen. Die von CONWAY angegebenen Gefässe haben wir nicht benutzt, weil man zur Alkalisierung mit K_2CO_3 das Gefäss öffnen muss. Bei den geringen Mengen ist es sicherer, auch die Kaliumkarbonatzugabe im geschlossenen Gefäss vorzunehmen. Aus diesem Grunde benutzten wir das in Abb. 1 dargestellte Gefäss, ein Erlenmeyerkölbchen mit Schliffaufsatz. Vor dem Gebrauch erhitzt man das Gefäss mit dem Porzellanschälchen im Trockenschrank auf 100°C, lässt auf 20°C abkühlen, pipettiert 4 ml Nessler auf den Boden des Erlenmeyerkölbchens, 0,5 ml Extrakt in das Porzellanschälchen und verschliesst das Gefäss mit der Schliffkapillare, die das K_2CO_3 enthält. Durch Zusatz der Kaliumkarbonatlösung zum Extrakt wird die Reaktion ausgelöst. Nach einer Reaktionszeit von 30 min bei 20°C entfernt man das Porzellanschälchen, schüttelt das Nesslersche Reagenz mehrfach um und liest nach genau 5 min im Photometer Eppendorf die Extinktion ab.

EICHWERTE
(Bezogen auf μg N)

μg Ammoniak–N:	$\frac{1}{2}$	1	2	3
Mittelwert:	0,0298	0,0598	0,122	0,178
Schwankung:	0,028–0,031	0,054–0,066	0,111–0,132	0,167–0,195
Anzahl der Messungen:	18	22	12	20
Leerwert:	0,072	0,073	0,085	0,071

Mittelwert für 1 μg Ammoniak–N: $\Delta E = 0{,}060$
Eichsubstanz: getrocknetes $(NH_4)_2SO_4$

Die Eichung wurde bis zu 5 μg Ammoniak–N für 4 ml 1 : 50 verdünnte Nesslersche Lösung geprüft. Die Extinktionszunahme verläuft linear, aber oberhalb 3 μg Ammoniak–N pro Ansatz beginnt das Reagenz trüb auszufallen. Es ist deshalb ratsam, nicht mehr als 3 μg Ammoniak–N pro Ansatz zu verwenden.

Als Mass für die Brauchbarkeit der Extraktgewinnung mit M/3 Perchlorsäure und der Bestimmungsmethode haben wir die Erhaltung der labilen Aminogruppe im Glutamin und Asparagin gewählt (HARRIS, 1943). Lösungen dieser Substanzen wurden dem gesamten Enteiweissungsprozess und der anschliessenden NH_3-Bestimmung unterworfen.

Wir finden eine Extinktionszunahme, die der Freisetzung von 0,12% der labilen Aminogruppe des Asparagins und 1,2% der labilen Gruppe des Glutamins entspricht. Löst man Glutamin direkt in M/3 Perchlorsäure und inkubiert 12 Stunden bei Zimmertemperatur von 20°C, so sind etwa 10% der labilen Aminogruppe als NH_3 freigesetzt.

ERGEBNISSE

1. *Gehirn* (Abb. 2a u. b, Tab. 1)

Die Versuche am Gehirn unterscheiden sich einerseits von den Versuchen an den anderen Organen durch die Gegenüberstellung der Veränderungen in akuter Ischämie und Anoxie und andererseits durch die Möglichkeit, das Gehirn ohne ischämische oder anoxische Belastung *in situ* einfrieren zu können. Man kann durch das Einfrieren *in situ* Veränderungen der Metabolitkonzentrationen abgrenzen, die durch die Gewebsentnahme selbst entstehen (Normaltier). Der freie Ammoniak gehört in

die Reihe der Metabolite, die ihre Konzentration bereits unter dem Reiz der Herausnahme verändern (KERR, 1942; RICHTER u. DAWSON, 1948; THORN *et al.*, 1955).

Wir finden am Normaltier (Tabl. 1, Abb. 2a u. b) im Mittel einen Gehalt an freiem Ammoniak von 0,23 mmol/l. bei 37°C und 0,1835 mmol/l. bei 26°C Körpertemperatur.

Unter dem Reiz der Herausnahme steigt der Gehalt bei normaler Körpertemperatur auf 0,4 mmol/l. im Mittel und auf 0,243 mmol/l. bei 26°C. Der Zuwachs in beiden Reihen verhält sich etwa wie 3 : 1.

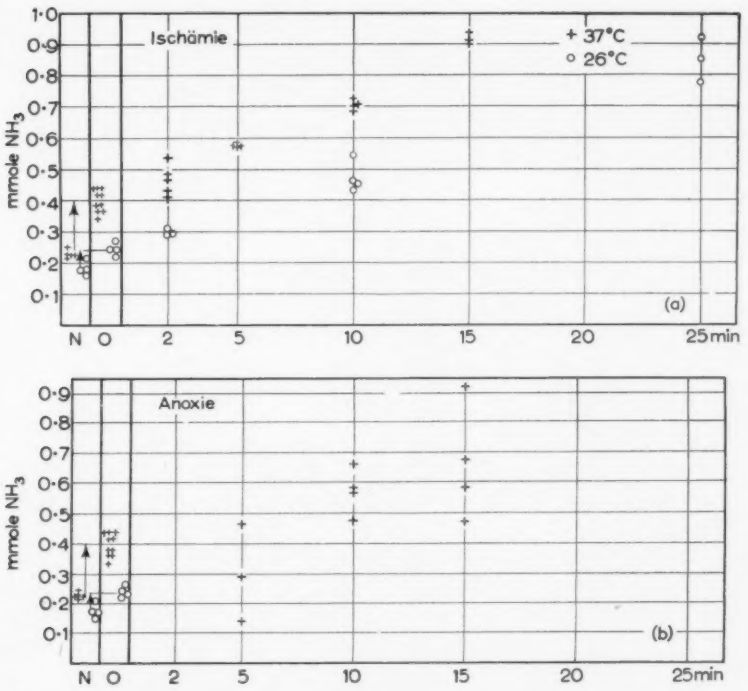

ABB. 2.—(a, b) Verhalten der Konzentration an freiem Ammoniak im Gehirn während der Ischämie (2a) und Anoxie (2b). Die Spalte N (Normaltier) enthält Werte für Gehirne, die *in situ* eingefroren wurden, O (Nulltiere) Werte für Gehirne, die mit einem vorgekühlten Löffel herausgenommen und in die flüssige Luft geworfen wurden. Die Pfeile deuten die Konzentrationszunahme unter dem Einfluss des Decerebrierens an. Alle Angaben in mmol/l.

In der Ischämie steigt der Gehalt nach 2 min auf 0,47, nach 5 min auf 0,5735 mmol/l. und auf 0,915 mmol/l. nach 15 min. Man kann den Anstieg für Versuchszeiten bis 15 min Ischämie bei 37°C mit Sicherheit als linear bezeichnen. Die mittlere Zunahme nach 2 min beträgt 0,035 mmol/l., nach 5 min 0,0346 mmol/l., nach 10 min 0,0309 mmol/l. und nach 15 min 0,0343 mmol/l. pro Minute (Mittel 0,0337 mmol/l. pro Minute).

Bei 26°C liegt ebenfalls ein linearer Anstieg des Ammoniak-Gehaltes für Ischämiezeiten bis 25 min vor. Für den mittleren Anstieg erhält man den Wert 0,0248 mmol/l. pro Minute.

Während sich die Konzentrationsunterschiede Normaltier/Nulltier bei 37°C

TAB. 1.—GEHALT AN FREIEM AMMONIAK IM GEHIRN*

37°C					
Normaltier	Nulltier	Ischämie			
		2 min	5 min	10 min	15 min
0,218–0,25 (5) 0,23	0,34–0,4 (10) 0,4	0,417–0,543 (5) 0,47	0,57–0,584 (4) 0,573	0,694–0,73 (4) 0,709	0,9–0,935 (3) 0,915

26°C				
Normaltier	Nulltier	Ischämie		
		2 min	10 min	25 min
0,164–0,213 (4) 0,1835	0,288–0,265 (4) 0,243	0,292–0,307 (3) 0,297	0,435–0,548 (4) 0,475	0,778–0,924 (3) 0,851

37°C			
	Anoxie		
	5 min	10 min	15 min
	0,144–0,46 (3) 0,298	0,479–0,665 (4) 0,5755	0,472–0,924 (4) 0,664

* Alle Angaben in mmol/l.

und 26°C wie 3 : 1 verhalten, ergibt sich für den Anstieg in der Ischämie der beiden Versuchsreihen ein Verhältnis von 1,4 : 1.

Die unter Stickstoffbeatmung ermittelten Werte (Abb. 2b) am Gehirn zeigen auch die Tendenz eines Anstiegs der Konzentration an NH_3. Die Streuung bei Tieren mit gleicher Belastung ist aber erheblich grösser als in der Ischämie. Nach 5 min Anoxie ist die Situation noch unübersichtlich. Erst nach 10 min Anoxie liegt ein eindeutiger Anstieg des Ammoniak-Gehaltes vor.

2. *Leber und Niere* (Abb. 3a u. b, Tab. 2)

Für die untersuchten Lebern (Nulltiere) fehlt der Vergleich mit *in situ* eingefrorenen Organen. Wir finden am Nulltier in der Leber bei 37°C 0,498–0,595 mmol/l. In der Asphyxie steigt der Gehalt an NH_3 und erreicht zwischen 5 und 15 min maximale Werte um 1,8 mmol/l. Die unteren Werte bei diesen Versuchszeiten liegen um 1,0 mmol/l. NH_3. In der Asphyxie bei 26°C ist nach 2 min noch keine Veränderung des NH_3-Gehaltes gegenüber dem der Nulltiere eingetreten. Nach 10 und 25 min werden Werte von rund 1,08 und 0,89 mmol/l. gefunden. Die nach anoxischer Belastung untersuchten Lebern zeigen ein uneinheitliches Bild, an dem die Zunahme der Streuung am meisten auffällt (Abb. 3b).

Nachdem wir genügende Erfahrung mit der Herausnahme von Herz und Leber hatten, haben wir versucht, auch noch die Niere in kurzer Zeit zu entnehmen und

einzufrieren. An 13 einwandfrei gewonnenen Nieren wurden NH_3-Bestimmungen durchgeführt, die in Tab. 2 zusammengefasst sind. Am Nulltier bei 37°c finden wir im Mittel 0,355 mmol/l. mit einer sehr kleinen Streuung und bei 26°c im Mittel 0,283 mmol/l.

In der Asphyxie schwanken die Werte nach 5 min bei 37°c zwischen 0,478–1,01 mmol/l.

In der Anoxie nach 5 min bei 37°c ergeben 2 Versuche 0,595 und 1,76 mmol/l. und nach 10 min bei einem Tier 1,02 mmol/l.

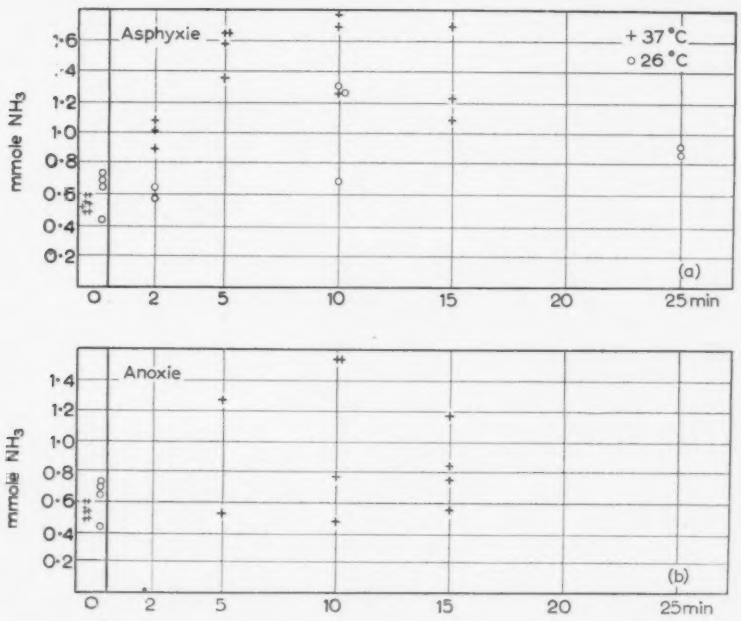

Abb. 3.—(a, b) Wiedergabe von NH_3-Konzentrationen an unbelasteten Lebern O (Nulltier) und während Asphyxie und Anoxie.

3. *Herz und Skelettmuskel* (Tab. 2, Abb. 4a u. b)

Die höchsten Werte für den Gehalt an freiem Ammoniak und die grössten Konzentrationsunterschiede finden wir bei unseren Versuchen im Herz- und Skelettmuskel. Im Herzmuskel der Nulltiere schwanken die ermittelten Konzentrationen zwischen 0,523–1,725 mmol/l. bei normaler Körpertemperatur. Bei 26°c ergeben sich Werte um 1,25 mmol/l., NH_3-Konzentrationen, die alle bereits höher liegen als die Werte im Gehirn nach 15 min Ischämie bei 37°c.

Es ist auffallend bei den Befunden am Herzen, dass weder nach 5 min Anoxie noch nach 5 min Asphyxie bei 37°c der Gehalt an NH_3 über die Schwankungen der Nulltiere hinausreicht, obwohl der Herzmuskel innerhalb dieser Zeit ein Maximum an anaerober Arbeit bewältigt hat (Scholl u. Thorn, 1957; Thorn *et al.*, 1957*b*). (Der Gehalt an Milchsäure ist bis zum Fünffachen angestiegen, an Phosphokreatin werden noch etwa 25 prozent des Ausgangswertes gefunden.)

In der Asphyxie werden erst nach 15 min bei 37°c und nach 25 min bei 26°c

TAB. 2.—DER GEHALT AN FREIEM AMMONIAK IN DEN EINZELNEN ORGANEN NACH VERSCHIEDENER BELASTUNG*

	Nulltier 37°C	Asphyxie 37°C				Anoxie 37°C		
		2 min	5 min	10 min	15 min	5 min	10 min	15 min
Leber	0,498–0,595 (7) 0,542	0,911–1,08 (3) 1,004	1,375–1,665 (4) 1,57	1,28–1,8 (3) 1,59	1,095–1,7 (3) 1,345	0,536–1,28 (2)	0,486–1,54 (4)	0,554 –1,17 (4)
Muskulatur	0,275–0,346 (4) 0,298	0,583–1,76 (3)	0,73–1,82 (5)	0,28–0,998 (4)	0,275–0,778 (3)	0,1215–0,265 (3)	0,357–1,92 (3)	0,0955–0,272 (4)
Herz	0,523–1,725 (4)		0,821–1,55 (3)		1,14–3,4 (3)	1,165–1,31 (3)	1,155 (1)	
Niere	0,348–0,358 (3) 0,355		0,478–1,01 (3)			0,595–1,76 (2)	1,02 (1)	

	Nulltier 26°C	Asphyxie 26°C			Anoxie 26°C		
		2 min	10 min	25 min	10 min	15 min	25 min
Leber	0,438–0,723 (4) 0,629	0,571–0,645 (2) 0,608	0,68–1,30 (3) 1,08	0,875–0,91 (2) 0,892			
Muskulatur	0,158–1,63 (4)	0,352–1,495 (4)	0,109–0,584 (3)	0,061–0,194 (3)			
Herz	1,135–1,4 (3) 1,25			1,155–2,51 (2)	2,50–3,15 (3) 2,93	2,48–3,27 (2) 2,87	2,39 –3,49 (4) 2,995
Niere	0,246–0,322 (3) 0,283						

* Alle Angaben in mmol/l.

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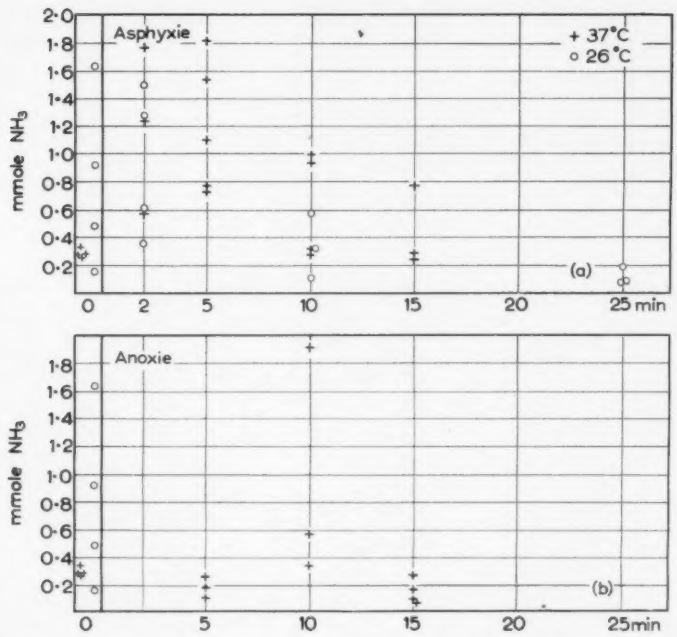

ABB. 4.—(a, b) Der Gehalt an freiem Ammoniak in der Muskulatur von Nulltieren bei 37°C und 26°C, in Asphyxie und Anoxie.

NH_3-Konzentrationen über 2 mmol/l. gemessen. In der Anoxie bei 26°C liegen die Werte etwa von der 10. min an um 2,5 mmol/l. und höher.

In der Muskulatur der Nulltiere finden wir bei 37°C einen recht niedrigen NH_3-Gehalt von 0,298 mmol/1. mit geringer Schwankung. Bei den unterkühlten Nulltieren dagegen findet man eine Schwankung um einen Faktor 10, der niedrigste Wert liegt bei 0,158 und der höchste bei 1,63 mmol/l. Diese Konzentrationsunterschiede erklären sich dadurch, dass die Hypothermie ein starker Weckreiz ist und in der Muskulatur unterschiedlich starke fibrilläre Zuckungen auslöst. Während der Asphyxie nimmt die Konzentration an freiem Ammoniak wieder ab, und nach 25 min bei 26°C werden noch 0,061–0,194 mmol/l. gefunden.

Bei 37°C haben wir einen niedrigen, einheitlichen Ausgangswert. In den ersten Minuten der Asphyxie steigen die Werte unter erheblicher Zunahme der Streuung bis auf 1,82 mmol/l. und fallen später wieder ab. Für diese Veränderungen in den ersten Minuten der Manschettenversuche bei 37°C sind die Krämpfe der Muskulatur verantwortlich, die heftiger einsetzen als bei der Stickstoffbeatmung (OPITZ u. THORN, 1949). Dementsprechend finden wir auch in der Anoxie bei 37°C keine derartigen Konzentrationsveränderungen.

DISKUSSION

Eine auf Einzelheiten zielende Diskussion der vielen Arbeiten, die dem Problem der NH_3-Freisetzung im Gewebe gewidmet sind, würde den Rahmen übersteigen. Bei kritischer Betrachtung kann man gegen eine Reihe dieser Publikationen zwei Einwände erheben:

Entweder wurden die Tierversuche einschliesslich der Gewebsgewinnung

unbefriedigend durchgeführt, oder man kann gegen die Extraktgewinnung und den Gang der Bestimmung Bedenken geltend machen, weil sie für die Fragestellung nicht glücklich gewählt waren.

Wenn man eine Wertung unter dieser Sicht anstrebt, so sind die Versuche von RICHTER u. DAWSON (1948) unter Berücksichtigung beider Gesichtspunkte am besten durchgeführt; denn jedes Gewebe, das nicht sofort eingefroren wird, gibt verfälschte NH_3-Werte durch inzwischen abgelaufene Desaminierungen.

Welche Aussagen sind auf Grund der vorliegenden Untersuchung möglich? Am gut durchbluteten, nicht belasteten Gehirn liegt der Gehalt an NH_3 bei normaler und herabgesetzter Körpertemperatur zwischen 0,15 und 0,25 mmol/l. mit einer auffallend geringen Schwankung (Normaltier). Unter dem Reiz des Decerebrierens steigt der Gehalt in Abhängigkeit von der Körpertemperatur plötzlich an. Damit ist ein alter Befund bestätigt, dass das Nervengewebe auf Reizung unter anderem mit NH_3-Freisetzung reagiert (RÖSCH u. TECAMP, 1928; WINTERSTEIN, 1933) Das Ausmass der Freisetzung ist von der Körpertemperatur abhängig (Abb. 2a).

Auch in der Muskulatur tritt bei Reizung eine NH_3-Freisetzung ein (PARNAS, 1932; PARNAS u. LUTWAK-MANN, 1935). Der ruhende Skelettmuskel hat einen Ammoniak-Gehalt von 0,27–0,35 m-mole/l. Unter dem Einfluss der tonisch-klonischen Krämpfe, die im Beginn der Manschettenversuche ablaufen, steigt der Wert um einen Faktor 6 an.

Während der Unterkühlung auf 26°C treten anfangs allgemeines Kältezittern, später ein erhöhter Muskeltonus mit fibrillären Zuckungen auf. Diese Erscheinungen dürften die Ursache für die Schwankungen in NH_3-Gehalt der Muskulatur bei 26°C am Nulltier sein (0,158–1,63 mmol/l.). Durch zusätzliche Urethangabe haben wir versucht, den starken Weckreiz, den die Kälte auf die Tiere ausübt, auszugleichen, um auch für 26°C den Ammoniak-Gehalt der ruhenden Muskulatur zu erfassen. Wie die Schwankung der Werte zeigt, ist das Vorhaben aber nicht geglückt.

Stellt man den Werten der Ischämieversuche am Gehirn (linearer Anstieg der NH_3-Konzentration mit geringer Schwankung) die Werte aus den Anoxieversuchen am Gehirn, an den anderen Organen und aus den Asphyxieversuchen gegenüber, so fällt auf, dass in Anoxie und Asphyxie der Gehalt an NH_3 stark streut. Nach 5 min Anoxie am Gehirn und nach 5 min Asphyxie am Herzen ist der NH_3-Gehalt gegenüber dem der Nulltiere nicht verändert, obwohl beide Organe sehr hohe anaerobe Stoffwechselaktivitäten besitzen. Deutliche Erhöhungen der NH_3-Konzentration treten im Gehirn und im Herzen erst nach 10, 15 und 25 min Versuchszeit auf. Da wir von denselben Tieren die Blutdruckhöhe während der Versuche kennen und das EKG abgeleitet haben (SCHOLL u. THORN, 1957), können wir aus der Höhe des Blutdrucks auf eine relativ gute Durchblutung der Organe bis zu 4 min schliessen. Später sinkt der Druck rasch auf 20 mm Hg und weniger. Wir möchten annehmen, dass die Durchblutung vermindernd auf den NH_3-Gehalt einwirkt. Erst wenn der Kreislauf völlig ausfällt, beginnt der NH_3-Anstieg. Unter den Bedingungen einer vollständigen Kreislaufunterbrechung werden sich alle Organe wie das Gehirn in der Ischämie verhalten und einen kontinuierlichen NH_3-Anstieg zeigen.

Auf Grund unserer Befunde können wir keine Stellung dazu nehmen, aus welcher Quelle die NH_3-Freisetzung gespeist wird. Mehrfach sind das Adenylsäuresystem und das Glutamin diskutiert worden (PARNAS u. LUTWAK-MANN, 1935; WEIL-MALHERBE, 1953; VRBA, 1955). Man kann den Annahmen entgegenhalten, dass

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die NH_3-Konzentration immer niedriger gefunden wird als aus der Konzentration der genannten Substanzen zu erwarten wäre. Eine andere unbeantwortete Frage ist, ob die plötzliche NH_3-Abspaltung bei einer Reizung des ZNS (hier durch das Decerebrieren) aus demselben Vorgang stammt wie der langsame, lineare, temperaturabhängige Anstieg in der Ischämie.

Es ist zur Zeit auch nicht klar, wie weit der NH_3-Abspaltung ammoniakbindende Reaktionen parallel laufen. Weil die bisherigen Angaben über den Gehalt an freiem Ammoniak im Gewebe recht unterschiedlich sind, wollten wir zunächst einmal nur die zu erwartenden Konzentrationen in den einzelnen Organen erfassen. Zur Klärung der angedeuteten Fragen bedarf es weiterer systematischer Untersuchungen.

ZUSAMMENFASSUNG

Unter Ausnutzung der von CONWAY angegebenen Diffusionsmethode zur NH_3-Bestimmung wird ein Reaktionsgefäss beschrieben, das die Austreibung des freien Ammoniaks aus dem einpipettierten Extrakt im geschlossenen System gestattet. Mit Hilfe dieser Methode wurden Bestimmungen der Konzentration an freiem NH_3 an Extrakten aus Gehirn, Leber, Herz, Muskulatur und Niere durchgeführt. Die Extrakte stammen von Kaninchen, die ischämischen, anoxischen und asphyktischen Belastungen ausgesetzt waren.

Am in situ eingefrorenen Gehirn wird ein Gehalt von 0,23 mmol/l. im Mittel bei 37°C und von 0,1835 mmol/l. bei 26°C gefunden. Unter dem Einfluss des Decerebrierens schnellt der Wert auf 0,40 mmol/l. bei 37°C und auf 0,243 mmol/l. bei 26°C. Während der Ischämie steigt der NH_3-Gehalt bei 37°C linear um 0,0337 mmol/l. pro min während der untersuchten Zeit bis zu 15 min und bei 26°C um 0,0248 mmol/l. pro min im untersuchten Bereich bis 25 min.

Der Gehalt an freiem Ammoniak beträgt in der Leber bei 37°C, 0,542 mmol/l., bei 26°C 0,629 mmol/l. Im Herzen schwanken die gefundenen Werte zwischen 0,52 und 1,72 mmol/l. bei 37°C und 1,13 bis 1,40 mmol/l. bei 26°C. In der Muskulatur stimmen die Werte bei 37°C gut überein, man findet 0,275–0,346 mmol/l. Bei 26°C dagegen streuen sie von 0,158–1,63 mmol/l. In der Niere findet man ohne Belastung 0,348–0,358 mmol/l. bei 37°C, 0,246–0,322 mmol/l. bei 26°C.

Während der Anoxie steigt der NH_3-Gehalt im Gehirn ebenfalls an, aber unter Zunahme von Konzentrationsschwankungen. Ein ähnliches Bild ergibt die Einwirkung von Asphyxie und Anoxie an Leber, Herz und Skelettmuskel.

Über die Quelle, aus der der freigesetzte Ammoniak stammt, kann nichts ausgesagt werden. Möglichkeiten werden diskutiert.

SUMMARY

An apparatus is described for estimating by CONWAY's method the free ammonia of a tissue extract in a closed system. The free ammonia in extracts of brain, liver, heart, muscle and kidney were determined by this method. The tissue extracts were obtained from rabbits under ischaemic and anoxic conditions and in asphyxia.

Brains frozen *in situ* gave mean of 0·23 m-moles per l. free NH_3 at 37°C, and 0·1835 m-moles/l. at 26°C. As a result of decerebration the free NH_3 rose to 0·4 m-moles/l. at 37°C, and to 0·243 m-moles/l. at 26°C. At 37°C and in ischaemia the ammonia content increased linearly at the rate of 0·0337 m-moles/l. min during the

observed period of 15 min; at 26°C the rate of increase was 0·0248 m-moles/l. min during the 25 min period studied.

The content of free ammonia in the liver was 0·542 m-moles/l. at 37°C, and 0·629 m-moles/l. at 26°C. In the heart the concentrations varied between 0·52 and 1·72 m-moles/l. at 37°C, and between 1·13 and 1·4 m-moles/l. at 26°C. In the musculature the scatter of values at 37°C was small (0·275 to 0·346 m-moles/l). However, at a temperature of 26°C they ranged from 0·158 to 1·63 m-moles/l. In the kidney there were found 0·348 to 0·358 m-moles/l. at 37°C, and 0·246 to 0·322 m-moles/l. at 26°C under normal conditions.

During anoxia the NH_3 concentration in the brain increased, but there was also an increased scatter in the values. In liver, heart, and skeletal muscle, similar values were found in asphyxia and in anoxia.

The source of ammonia is unknown. Various possibilities are discussed.

ANHANG

Die gefundenen Unterschiede in der Ammoniakkonzentration werden unter Errechnung der Standardabweichung mit Hilfe der *t*-Verteilung auf ihre Signifikanz geprüft.

	arith. Mittel	Standardabw.
(1) Normaltiere 37°c	0,230	0,012
(2) Nulltiere 37°c	0,400	0,121
(3) Normaltiere 26°c	0,184	0,021
(4) Nulltiere 26°c	0,243	0,016

		t	*F*	Sicherungsgrenze
Vergleich der Mittelwerte:	(1) u. (2)	4,43	13	0,1%
	(1) u. (3)	3,97	7	1%
	(2) u. (4)	4,00	12	0,27%
	(3) u. (4)	4,50	6	1%

Einwandfrei signifikante Unterschiede ergeben sich einmal für den Gehalt an freiem Ammoniak im Gehirn zwischen Normalund Nulltieren, zum anderen für die Differenz in der NH_3- Konzentration bei normaler und herebgesetzter Körpertemperatur.

F = Anzahl der Freiheitsgrade

t = Masszahl für die Abweichungen.

Sicherheitsgrenze ergibt sich aus den Sicherheitsstufen der *t*-Verteilung.

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HISTOCHEMICAL MECHANISMS OF THE MARCHI REACTION FOR DEGENERATING MYELIN

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(*Received* 8 *July* 1957)

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THE essential principle of the Marchi method is the reduction of osmium tetroxide (OsO_4) to the black lower oxide, osmium dioxide, by myelin. This reduction occurs not only with normal, but also with degenerating myelin. The reduction of OsO_4 by normal myelin may be inhibited by the addition of potassium dichromate (MARCHI, 1886) or chlorate to the solution of OsO_4. It has been customary to apply the terms Marchi-negative and Marchi-positive to normal and degenerating myelin respectively.

The chemical basis of the Marchi method has never been satisfactorily elucidated. For this reason it is proposed, in this paper, to study the physicochemical aspects of the reaction. JOHNSON, MCNABB and ROSSITER (1950), CAIN (1950) as well as NOBACK and MONTANA (1952) were unable to explain the selective inhibition of the reducing properties of normal myelin for osmium tetroxide (OsO_4) brought about by the addition of chlorate or dichromate, when contrasted with the failure of these oxidizing agents to prevent the blackening of degenerating myelin by OsO_4. For this reason these authors dismissed the Marchi method as a technique of little value in biochemical or histochemical studies of demyelination.

A further difficulty arose in that when the reagents were applied in separate stages, i.e. the tissues were first oxidized by chlorate before they were stained by OsO_4, there was no resulting diminution in the blackening of normal myelin. It was necessary, in fact, to add a suitable oxidizing agent to the OsO_4 solution in order to suppress the reaction in normal myelin and allow selective staining of degenerating myelin (SWANK and DAVENPORT, 1934).

WOLMAN (1956) has suggested that the positive staining of degenerating myelin by the Marchi method is due to a compound 'having some chemical characteristics in common with heparin'; later (1957) he identified this compound as an acid mucopolysaccharide. This view does not, however, take into account that degenerating myelin is soluble in acetone (HURST, 1925) and therefore behaves more like a lipid than a carbohydrate.

A feature of the Marchi method, not previously appreciated, is that the reagents employed in the method have quite different solubilities. Thus polar compounds such as chlorate or dichromate are soluble only in aqueous media, whereas OsO_4, as will be shown later, is quite strikingly soluble in all types of lipid. Now, normal myelin is a substance which has an affinity for water and can be stained by water-soluble dyes such as neutral red. Conversely, degenerating myelin is unstained by aqueous dyes, but readily absorbs lipophilic dyes, such as the Sudans.

The affinity of normal myelin for water is probably related to the presence of phospholipids* which rapidly absorb water to form surface-outgrowths known as myelin-buds (VIRCHOW, 1854; LEATHES, 1925; SCHMITT and PALMER, 1940; HÖBER, 1945): phospholipids can also form colloidal suspensions in water (HEILBRON and BUNBURY, 1943; HÖBER, 1945; FRAZER, 1953). Now the absorption of water by phospholipids may indicate that the myelin sheath is permeable to water-soluble oxidizing agents such as chlorate, and from this it could be postulated that the negative result of normal myelin with Marchi's method is due to the prevention of the reaction $OsO_4 \rightarrow OsO_2$ by chlorate.

If this is correct the question arises: how does chlorate suppress the reduction of OsO_4? This problem is clarified by the work of ZELIKOFF and TAYLOR (1950) who showed that chlorate, in the presence of OsO_4, oxidized the double bonds of fumaric and maleic acids more rapidly than in the absence of OsO_4. Since OsO_4 was not reduced in this reaction it was concluded that the role of OsO_4 in an OsO_4-chlorate mixture was that of a catalyst or a 'carrier.' This view was corroborated by HOFFMANN's (1913) observation, which I have confirmed, that the oxidation–reduction potential of an OsO_4-chlorate mixture was higher than that of either reagent alone. Accordingly it may be said that OsO_4, although not itself reduced, increased the 'oxidizing power' of chlorate. Since fumaric and maleic acids were, in effect, Marchi-negative it is possible that this catalytic, as opposed to oxidizing, role of OsO_4 may also explain the negative result of normal myelin with Marchi's method.

The purpose of this paper is to show that the absorption of chlorate by normal myelin is the factor responsible for the lack of staining of normal myelin with the Marchi method and, conversely, that the blackening of degenerating myelin is due to the impermeability of the myelin breakdown products to chlorate.

METHODS AND RESULTS

Experiments in vitro. Normal myelin lipids, used in these experiments, were either complete extracts or individual lipid samples (sphingomyelin, cerebroside and cholesterol). Lecithin, which is probably not a typical myelin lipid, was included in this group since it is an example of a phosphoglyceride. Olive oil (neutral fat), oleic acid (fatty acid) and cholesterol oleate (esterified cholesterol) were also used since they represent the three groups of lipids which, on theoretical grounds, could be formed by chemical degradation of myelin.

The following investigations were made on these preparations of normal and 'degenerating' myelin lipids:

(a) The property of forming myelin buds. It was assumed that lipids which form these buds have an affinity for water (hydrophilia) and, conversely, that lipids which do not form these buds repel water (hydrophobia).

(b) The reaction of each lipid with the Marchi method.

(c) The permeability of each lipid to OsO_4 and chlorate. Permeability to bromine and iodine were also studied since the extent of absorption of these halogens was relevant to their value as blockading agents for the double bonds of lipids (see 2a).

*Phospholipids account for approximately half the lipids of white matter. The lipids of mammalian white matter and peripheral nerve, i.e. those of myelin as well as those of the axon, are constituted as follows: 15 per cent phosphosphingosides (sphingomyelin), 35 per cent phosphoglycerides (kephalin) and 25 per cent each of glycosphingosides (cerebroside) and cholesterol. The foregoing figures, which are quite approximate have been based on data supplied by BRANTE (1949), FOLCH-PI (1955) and ROSSITER (1955). There is no ganglioside, neutral fat or esterified cholesterol in normal white matter (BRANTE, 1949; EDGAR, 1956).

Experiments with tissue sections. These were of two types:

(a) To confirm that the double bond of the fatty acid side-chain is the group responsible, in lipids, for the reduction: $OsO_4 \rightarrow OsO_2$.

(b) To demonstrate the presence of unreduced OsO_4 in normal myelin previously treated with OsO_4-chlorate.

Experiments in vitro

1(a) The capacity to form myelin buds was examined in the following lipids: lecithin (crude), sphingomyelin (purified), cerebroside (purified), myelin lipid samples (with and without extraction of proteolipid-protein by the method of FOLCH and LEES (1951), olive oil, oleic acid, cholesterol and cholesterol oleate. A sample of each lipid tested was spread as a thin streak on a glass slide, sufficient 0·9 per cent saline was added to cover the streak and then a cover slip was placed on top of the completed preparation. With each lipid one preparation was left at room temperature and another at 40–50°C. After one hour the edge of each streak was examined microscopically for the presence of myelin buds.

Lecithin and the myelin lipid samples (with (Fig. 1) or without extraction of protein) formed myelin buds readily at room temperature and even more abundantly at 40–50°C. With sphingomyelin and to a lesser extent with the cerebroside sample (Fig. 2) myelin buds were seen only at the higher temperature employed. Olive oil, oleic acid, cholesterol and cholesterol oleate did not show this property in saline at either high or low temperatures.

1(b) The lipids employed in the foregoing study were again spread as streaks on glass slides. Duplicate samples were stained for 18 hr either by 0·3% OsO_4 or by the 0·3% OsO_4—1% potassium chlorate mixture of SWANK and DAVENPORT (1935). All the lipids, except cholesterol, were blackened by OsO_4 alone. The myelin lipid extracts (with and without proteolipid-protein), sphingomyelin, lecithin, cerebroside and cholesterol were unstained by OsO_4-chlorate apart from a slight brown discoloration of all but the last. On the other hand olive oil, oleic acid and cholesterol oleate reduced OsO_4-chlorate and were stained jet-black.

1(c) Samples of the myelin lipid extract (proteolipid-protein removed), olive oil, oleic acid and cholesterol oleate were spread on glass so that at least one edge of the smear presented a straight edge. The reagents tested (0·3% OsO_4, 1% potassium chlorate in 0·01N-sodium hydroxide solution, saturated aqueous bromine and Lugol's iodine solution) were dropped on to each sample and a cover slip was pressed down firmly over the whole. After 1 hr the width of the black or coloured zone at the edge of each lipid was measured with a micrometer eye-piece: it was assumed that the width of this zone was proportional to the rate of reagent-absorption for the lipid in question. In the case of chlorate finely crushed crystals of pyrogallol were mixed with each sample of lipid and when permeable to chlorate a brown ring, formed by the oxidation of alkaline pyrogallol, was seen at the edge of that particular lipid. The rates of reagent-absorption for each of the four lipid samples are presented in the table. OsO_4 and bromine were absorbed rapidly by all the lipids tested; chlorate was only absorbed by the myelin lipids; iodine was absorbed well by the myelin lipids but only slowly by olive oil, oleic acid and cholesterol oleate. The results obtained with the myelin lipid extract are probably low since no account was taken of the gradual erosion of the lipid surface owing to the formation of myelin buds.

Experiments with tissue sections

2(a) Human material was obtained from 4 cases of multiple sclerosis, and murine from 8 mice whose sciatic nerves had been divided 12–16 days previously. Corresponding control tissues were taken from normal brain and cord in the human and, in mice, from the contralateral unsectioned nerve. Frozen sections* were stained by the OsO_4-chlorate mixture used by SWANK and DAVENPORT in their modification (1935) of the Marchi method (Fig. 3). Duplicate sections were stained by 0·3% OsO_4 without the addition of the chlorate of SWANK and DAVENPORT's fluid (Fig. 4). Preliminary treatment for 18 hr with either a saturated aqueous solution of bromine or with Lugol's iodine solution was applied to other sections in order to saturate the double bonds of lipids, and thus prevent the subsequent reduction of either OsO_4-chlorate or OsO_4 by these bonds.

* Owing to the number of different treatments applied to each piece of tissue, it was not practical to employ the usual method of block-impregnation of tissues with OsO_4-chlorate: LILLIE's (1954) method of staining frozen sections with the fluid was used instead.

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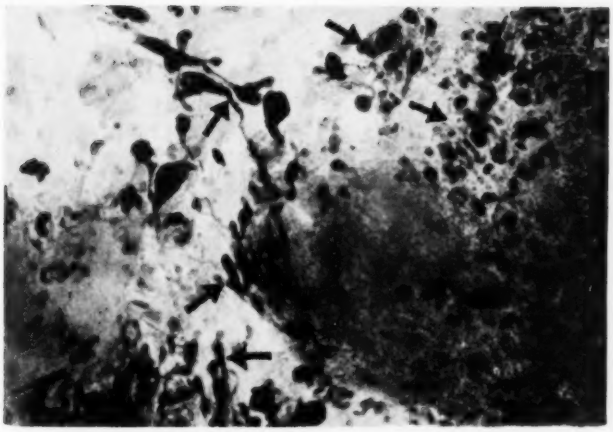

FIG. 1.—Large myelin buds (opposite arrows) at the edge of a streak of myelin lipid extract (protein-free), ×60.

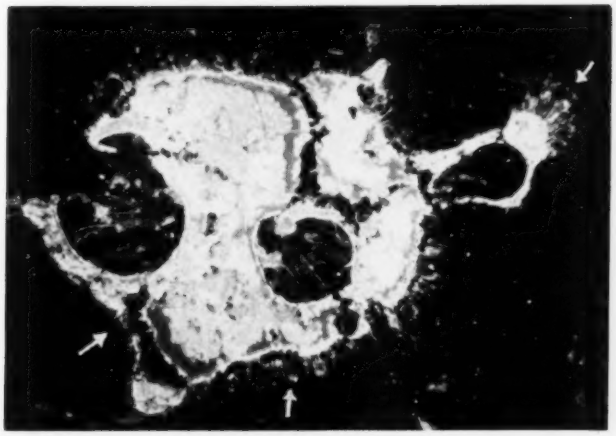

FIG. 2.—Fine fungal-like buds (opposite arrows) at the surface of a sample of cerebroside (40–50°C), dark ground, ×51.

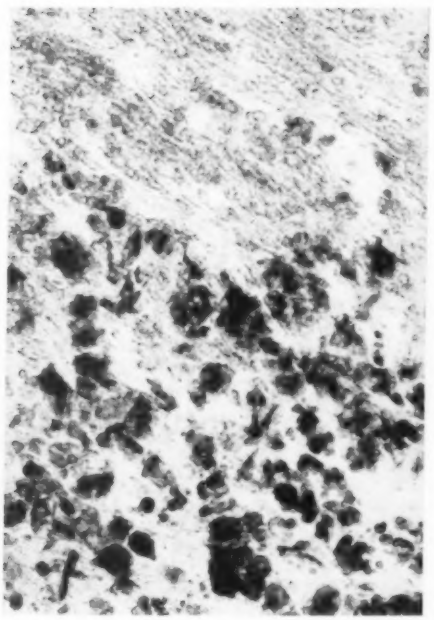

FIG. 3.—Multiple sclerosis: edge of a plaque of demyelination, stained by OsO_4-chlorate, ×410. Note selective staining of degenerating myelin (bottom half).

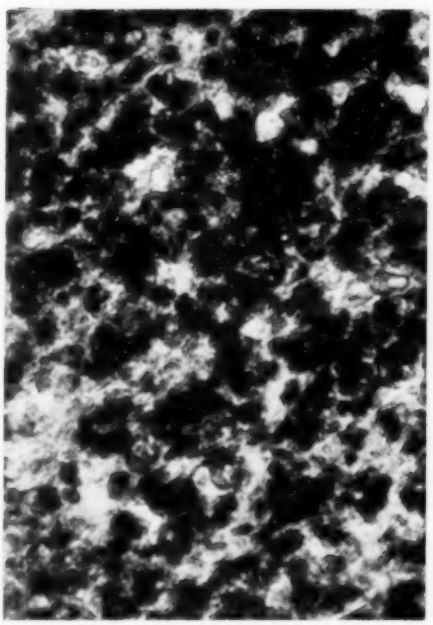

FIG. 4.—Same tissue as in Fig. 3, stained by OsO_4, ×420. Both normal (top half) and degenerating myelin (bottom half) are stained.

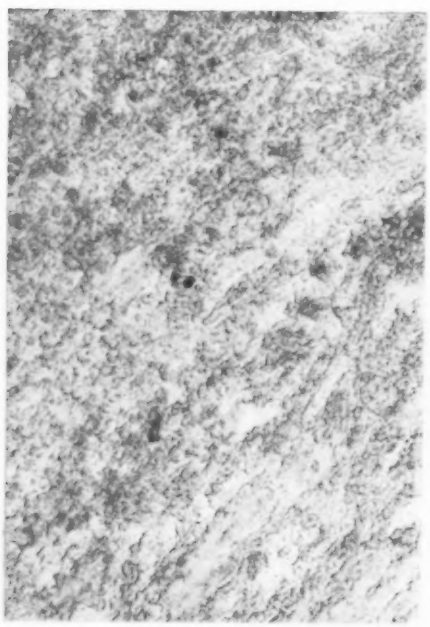

FIG. 5.—Same tissue and method as in Fig. 3, but with preliminary bromination, ×410. Degenerating myelin is now unstained.

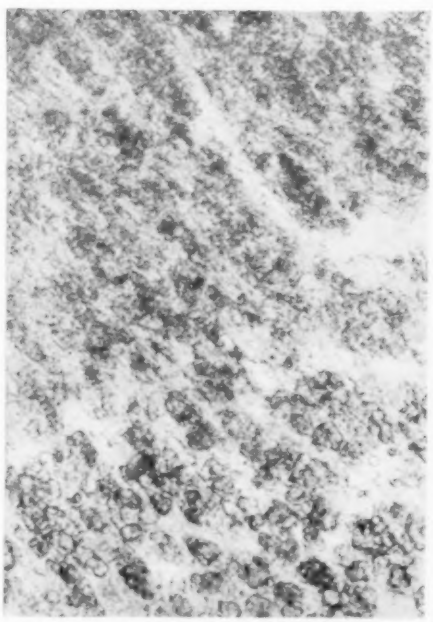

FIG. 6.—Same tissue and method as in Fig. 4, but with preliminary bromination, ×410. The reaction of all lipids has been suppressed.

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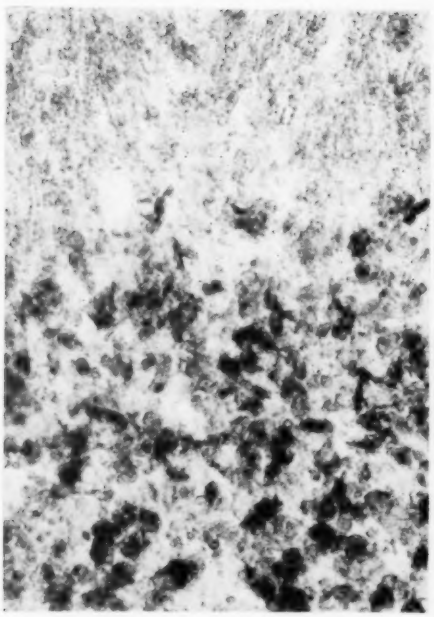

FIG. 7.—Same tissue and method as in Fig. 3, but with preliminary iodination, ×410. This iodination has not prevented the blackening of degenerating myelin.

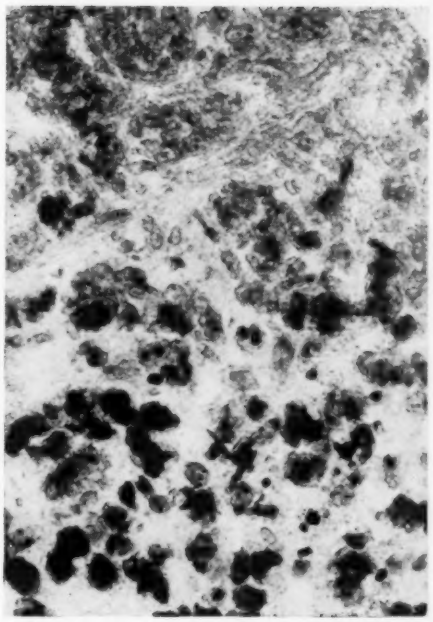

FIG. 8.—Same tissue and method as in Fig. 4, but with preliminary iodination, ×410. The staining of normal myelin by OsO_4 has been reduced to negligible proportions.

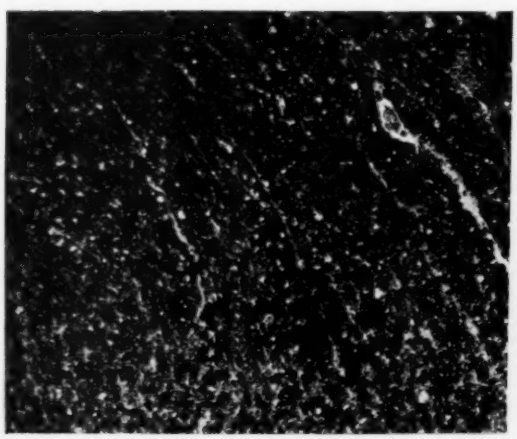

FIG. 9.—Similar tissue to Fig. 3, OsO_4-chlorate-α-naphthylamine, ×77, blue-green filter. Normal myelin (top half), which is stained red, and degenerating myelin (bottom half) both appear black with this filter.

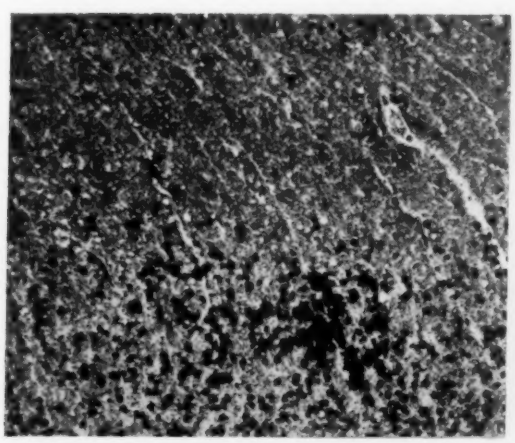

FIG. 10.—Same field and magnification as Fig. 9, but with red filter. The red colour of normal myelin has not been absorbed by this filter; normal myelin now appears pale whereas degenerating myelin is still black.

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To assess the extent of absorption of iodine by normal and degenerating myelin some sections were exposed for 18 hr to an aqueous solution of $^{131}I_2$ (6 mc per 100 ml). After thorough washing in 5% sodium thiosulphate solution and in distilled water the sections were coated with autoradiographic film (PEARSE, 1953) which was developed 4–6 hr later.

Bromination prevented the blackening of degenerating myelin (Fig. 5) and connective-tissue fat by OsO_4-chlorate. Likewise bromine inhibited the reduction of OsO_4 by normal and degenerating myelin (Fig. 6) and by fat droplets. Iodination, on the contrary, had no effect on the staining of either degenerating myelin (Fig. 7) or fat droplets by OsO_4-chlorate. Treatment with this halogen, however, suppressed the reduction of OsO_4 by normal myelin (Fig. 8). In sections treated with $^{131}I_2$ there was strong radioactivity in normal myelin, but no activity could be detected in the areas of degeneration where a positive reaction had been obtained, in near-adjacent sections, with OsO_4-chlorate.

2(b) In order to demonstrate the presence of 'unreduced' OsO_4 in normal myelin previously treated with OsO_4-chlorate, a histochemical adaptation of WINGFIELD and YOE's (1956) colorimetric method for osmium was devised. In this adaptation frozen sections (as for 2a) already stained by OsO_4-chlorate, were immersed for 1 hr in a saturated aqueous solution of α-naphthylamine at 37°c. Normal myelin was stained red (Fig. 9) whereas degenerating myelin was black (Fig. 10). Sections which had not been treated with OsO_4-chlorate were unstained by α-naphthylamine. Preliminary bromination prevented the blackening of degenerating myelin and reduced the intensity of the red colour in normal myelin to negligible proportions.

DISCUSSION

The chemical basis of the Marchi reaction was studied by histophysical and histochemical methods applied to lipids *in vitro* and in tissue sections.

Whole extracts of normal myelin lipids had a marked affinity for water in that they formed profuse myelin-buds. The purified samples of sphingomyelin and brain cerebrosides did not form these myelin-buds except at relatively high temperature, in contrast to lecithin and kephalins which exhibit this property at room temperature (SCHMITT and PALMER, 1940).

However, no matter which lipid fraction is responsible, complete myelin lipid extracts have the property of forming myelin-buds to a marked degree: the extraction of lipid-bound protein (the proteolipid-protein of FOLCH and LEES, 1951) in no way modified the formation of buds by the myelin lipids.

Lipids such as neutral fat, fatty acid and esterified cholesterol did not show myelin-bud formation. On this basis the myelin lipids on account of their affinity for water were termed hydrophilic, whereas fatty acids and their esters both with glycerol (neutral fat) and cholesterol were designated hydrophobic.

The separation of myelin lipid from myelin protein by organic solvents did not reverse the negative-Marchi reaction of the lipid extract. From this it is inferred that the physicochemical relationship of the protein layer to the lipid layer in the individual myelin lamella (FERNÁNDEZ-MORÁN, 1950; SCHMITT, 1950; FINEAN, 1953*a* and *b*) is not a factor involved in maintaining the hydrophilic state of myelin. Likewise extraction of lipid-bound proteolipid-protein did not result in blackening of the myelin lipids with OsO_4-chlorate. It is concluded that the hydrophilia of myelin is a characteristic of the myelin lipids rather than of myelin proteins or lipid-protein combinations.

Preliminary bromination suppressed the reduction of OsO_4, either alone or as OsO_4-chlorate, by both normal and degenerating myelin and by connective tissue fat as well. The success of bromine blockade indicated that double bonds were involved in the reduction of OsO_4 by these lipids: the double bonds were probably present

mainly in unsaturated fatty acids and aldehydes but, in part, in unsaturated sphingosine as well. In this connexion cholesterol—a lipid with no fatty acid side-chain—was not stained by either OsO_4 or OsO_4-chlorate. Iodination failed to prevent the reduction of OsO_4 and OsO_4-chlorate by degenerating myelin and by connective-tissue fat. Normal myelin, however, could not be blackened by OsO_4 after treatment with this halogen. Thus iodine was effective as a blockading reagent for hydrophilic lipids but not for the hydrophobic sorts. The failure of iodine to saturate the double bonds of hydrophobic lipids may be related to the relative insolubility of iodine in such lipids, as was shown *in vitro* (see Table 1) and by autoradiography, when compared with the greater solubility in them of bromine and OsO_4.

TABLE 1.—PERMEABILITY OF LIPIDS TO OsO_4, $KClO_3$, Br_2 AND I_2

	Absorption (mm/hr)			
	Myelin lipids	*Olive oil*	*Oleic acid*	*Cholesterol oleate*
OsO_4	0·92	0·48	0·58	0·52
Alkaline $KClO_3$	0·96	<0·01	saponified	<0·01
Br_2	1·20	0·58	0·53	*ca.* 0·50
I_2	0·66	0·16	0·16	0·14

Degenerating myelin is Marchi-positive when the process has reached the stage of chemical degradation: in the earlier phase of demyelination, when there is marked physical fragmentation but no chemical destruction of the sheath (JOHNSON *et al.*, 1950), the Marchi reaction is negative (NOBAK and MONTANA, 1952). There is no significant chemical change in the myelin lipids until early in the second week after section of the peripheral nerve (BRANTE, 1949; JOHNSON *et al.*, 1950), but thereafter the normal lipids are progressively destroyed and replaced by increasing amounts of esterified cholesterol.

Although GLEES (1948) has claimed that a few pyramidal fibres may be stained as early as 3 days after a cortical lesion in cats and monkeys, nevertheless it is usually accepted that degenerating myelin cannot be satisfactorily stained by Marchi's method until about the beginning (ROSSITER, 1955) to the middle of the second week after section of fibres in the mammalian C.N.S. (METTLER, 1932; METTLER and HANADA, 1942) or peripheral nerve (NOBAK and MONTANA, 1952). At the same time the droplets of degenerating myelin become sudanophilic and are stained pink by Nile blue sulphate (NOBAK and REILLY, 1956).

It would, therefore, be reasonable to postulate that the formation of hydrophobic cholesterol esters, which are foreign to the normal sheath, is the factor which determines the blackening of degenerating myelin by Marchi's solution. In support of this argument cholesterol oleate is stained orange-red by Sudan III, pink to mauve

by Nile blue sulphate and is soluble in acetone: all these properties have been described in degenerating myelin by HURST (1925) and were confirmed here.

The view has been held that neutral fat may be formed during demyelination: this hydrophobic lipid, if in fact formed, could also contribute to the blackening of degenerating myelin by OsO_4-chlorate. However, BRANTE (1949) found no evidence that myelin lipids were degraded to neutral fat, and CUMINGS found esterified cholesterol in 4 out of 5 cases of multiple sclerosis (1953, 1955) but no neutral fat (1955) in affected areas of white matter. Likewise RODNIGHT (1957), in two cases of multiple sclerosis, found a considerable amount of esterified cholesterol in areas of complete demyelination.

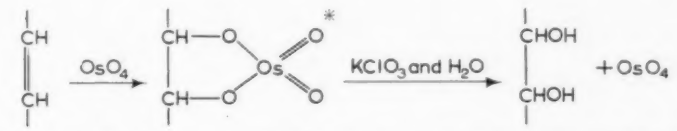

FIG. 11.—The role of OsO_4 as a catalyst for the oxidation of the double bond by chlorate (after ZELIKOFF and TAYLOR, 1950): the ethylene-OsO_4 addition compound (*) is oxidized by chlorate with re-formation of free OsO_4. (The oxidation of the ethylene group (—CH=CH—) of the unsaturated dicarboxylic acids, unlike that of unsaturated fatty acids, does not proceed as far as the formation of aldehyde.)

Normal myelin was stained red by α-naphthylamine after preliminary treatment of the sections with OsO_4-chlorate. Now α-naphthylamine, as its trisulphonic acid-derivative, has been used by WINGFIELD and YOE (1956) for the colorimetric estimation of OsO_4 as osmate (OsO_4^{2-}). It may be inferred, therefore, that OsO_4 is absorbed from OsO_4-chlorate by normal myelin, but is retained in a relatively oxidized state

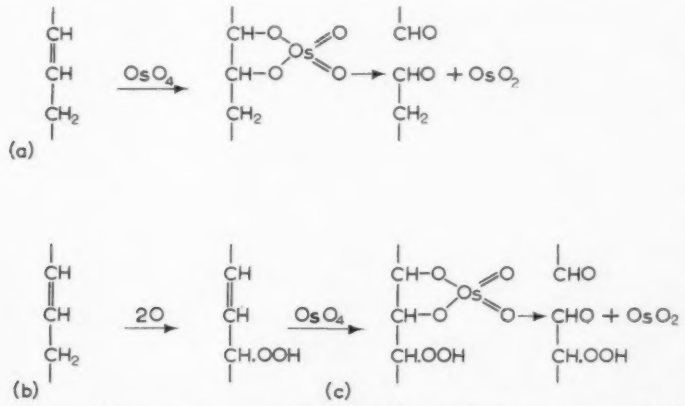

FIG. 12.—(a) Oxidation of an unsaturated fatty acid chain by OsO_4: note the addition of OsO_4 to the double bond. (b) Oxidation by chlorate: note the formation of hydroperoxide at the carbon atom adjacent to the double bond. (c) The double bond has survived as result of (b) leaving this group free to react with OsO_4.

as OsO_4^{2-} and remains colourless until treated with α-naphthylamine. The absence of such OsO_4^{2-} in normal myelin, which had first been subjected to bromination, suggested that the OsO_4^{2-} demonstrated by α-naphthylamine was in the form of an addition compound with double bonds (as in Figs. 11 and 12).

From my studies of myelin lipids *in vitro* it would be reasonable to infer that the

normal myelin sheath is permeable to chlorate. If so then the failure of the hydrophilic myelin lipids to reduce OsO_4, in an OsO_4-chlorate mixture, may be accounted for by the permeability of these lipids to chlorate. In this connection ZELIKOFF and TAYLOR (1950) attributed only a catalytic role to OsO_4 when mixed with chlorate: the rate of oxidation of fumaric and maleic acids by chlorate was increased but there was no corresponding reduction of OsO_4. These authors suggested that, at first, OsO_4 forms an addition compound with the double bond (Fig. 11). Subsequently chlorate oxidizes this addition compound to form a hydroxy-acid: OsO_4 is re-formed in the process. If ZELIKOFF and TAYLOR's conclusions are now applied to normal myelin then the failure of OsO_4-chlorate to stain the normal sheath may be attributed to the inhibition of the reduction of OsO_4 by the absorbed chlorate. Admittedly these authors were concerned with the double bonds of unsaturated *dicarboxylic* acids, but there is no reason to suppose that the reactions of an OsO_4-chlorate mixture with the unsaturated *fatty* acids of hydrophilic lipids would be significantly different.

It should be noted that preliminary oxidation of tissues with chlorate or dichromate in no way suppresses the Marchi reaction of degenerating myelin. In fact preliminary oxidation with dichromate was recommended in the classical Marchi method and also, with chlorate, in the GLEES modification (1943). Such preliminary oxidation would be expected to saturate all double bonds and thus destroy the reducing properties of degenerating myelin but, as the degeneration-products are of hydrophobic character, it is unlikely that chlorate or dichromate would gain sufficient physical access to the breakdown products to cause the saturation of double bonds therein.

On the other hand the failure of chlorate to prevent the blackening of the normal sheath by OsO_4 alone cannot be attributed to physical inaccessibility, since it was shown that normal myelin is permeable to chlorate. However it is likely that oxidation of an unsaturated fatty acid proceeds firstly by the formation of hydroperoxide at the carbon atom adjacent to the double bond while, at first, sparing the latter (DEBUCH, 1956). In support of this view it was shown here by means of the Nadi reaction that oxidation by chlorate, with the exclusion of atmospheric oxygen, resulted in the formation of peroxide in normal myelin. However this type of oxidation did not prevent the subsequent reduction of OsO_4 by myelin. Conversely no peroxide was found in normal myelin after oxidation by OsO_4-chlorate and, moreover, OsO_4 was not reduced by normal myelin after the latter type of oxidation. This finding is in conformity with ZELIKOFF and TAYLOR's (1950) conclusion that chlorate, when catalysed by osmium, oxidizes the double bond directly.

It is clear therefore that oxidation of unsaturated lipids by chlorate, with the formation of peroxide (Fig. 12b), is essentially different both to osmium-catalysed oxidation by chlorate, where the double bond is involved from the beginning (Fig.11), and also to the straightforward reduction of OsO_4 by these double bonds (Fig. 12a). Uncatalysed oxidation by chlorate would, in effect, leave the double bond free to be oxidized by OsO_4 (Fig. 12b and c). However, it is likely that prolonged uncatalysed oxidation does destroy the double bond, in as much as the iodine number of all lipids falls progressively when stored in contact with air.

There remains now the question of the origin of Marchi-artefacts, so fully described by SMITH (1956). *In vitro*, as previously stated, myelin lipids were not stained by OsO_4-chlorate. However, if these lipid extracts were first soaked in normal hydrochloric acid, patchy areas of blackening were then seen after treatment with

OsO_4-chlorate. Similar but rather weaker staining was noted after preliminary oxidation with mercuric chloride. This type of reaction, *in vitro*, may be due to the release of aldehydes from acetal phospholipids (plasmalogens), for mercuric chloride and hydrochloric acid may be used for this purpose in the plasmal reaction. Fatty aldehydes are hydrophobic and would accordingly fulfil the criterion for a positive-Marchi reaction.

The occurrence of Marchi-artefacts in tissues could not, however, be related to the formation of hydrophobic fatty aldehydes, since artefacts were not seen in sections of tissues which had been first treated, with either hydrochloric acid or mercuric chloride. On the other hand it is possible that artefacts may have been missed, since the intensity of staining with the method used, i.e. the application of OsO_4-chlorate to frozen sections, was less than with the classical method of impregnating the tissue, in block, with OsO_4-chlorate.

Although no staining of normal myelin with OsO_4-chlorate was encountered in the method used here, nevertheless blackening of some normal sheaths is not uncommonly seen after block-impregnation (SMITH, 1956). The cause of this type of artefact—the staining of the normal sheath—has not been elucidated.

It is concluded that the absence of blackening which is usually seen in the normal myelin sheath with the Marchi method is due to the hydrophilic properties of myelin: these hydrophilic properties are a characteristic of the myelin lipids rather than proteins. The blackening of degenerating myelin is ascribed to the formation of hydrophobic cholesterol esters by chemical degradation of myelin.

SUMMARY

(1) Normal myelin lipids are hydrophilic, Marchi-negative and absorb both OsO_4 and chlorate. On the contrary neutral fat, fatty acids and cholesterol esters of fatty acids are hydrophobic, Marchi-positive and are permeable to OsO_4 but not to chlorate.

(2) The separation of myelin lipids from myelin proteins by chemical extraction in no way alters the hydrophilic properties of these lipids.

(3) It was confirmed that OsO_4 is reduced by double bonds, probably mainly those of unsaturated fatty-acid side-chains.

(4) 'Unreduced' OsO_4 can be detected in normal myelin after treatment with OsO_4-chlorate: from chemical evidence it is likely that chlorate prevents the reduction of OsO_4 by double bonds.

(5) It is suggested that the negative-Marchi reaction of normal myelin is determined by the hydrophilic properties of the myelin sheath. Conversely the blackening of the myelin breakdown products is associated with the hydrophobic nature of degenerating myelin. In the former chlorate is absorbed and suppresses the reduction of OsO_4; in the latter, chlorate is not absorbed and accordingly there is no impediment to the straightforward reduction of OsO_4.

(6) The hydrophobic properties of degenerating myelin may be related to the formation of cholesterol esters during the chemical degradation of myelin.

Acknowledgements—I wish to thank Professor DOROTHY S. RUSSELL, Mr. D. J. ARNOTT, Dr. MARION C. SMITH and Dr. G. H. SLOANE-STANLEY for their help and advice in the preparation of this paper. I am also indebted to Mr. K. V. SWETTENHAM for technical assistance and to Mr. A. L. GALLUP for the photomicrographs.

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ON THE MECHANISM OF AMMONIA FORMATION IN GUINEA PIG BRAIN SLICES*

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(*Received* 22 *May* 1957)

SINCE 1922, when TASHIRO discovered that a nerve stimulated *in vitro* releases ammonia, many experiments have been published, showing that activity of nervous tissue is associated with increased formation of ammonia. This has been shown in experiments with isolated nerves *in vitro*, using various types of stimulation, e.g. electrical stimulation, stimulation by heat, light or mechanical damage, as well as stimulation by various chemical agents (TASHIRO, 1922; WINTERSTEIN and HIRSCHBERG, 1925; GERARD and MEYERHOF, 1927; ROSCH and TEKAMP, 1928; PRAWDICZ-NEMINSKI, 1932, 1933; HALTER, 1933; KAGANOVSKAYA and KAN, 1936).

Increased ammonia formation has also been observed in the central nervous system. Experiments *in vivo* have shown that the concentration of ammonia in the brain increases after stimulation by pharmacological agents and electric shock, and during hypoxia, unconditioned and conditioned reflex activity, etc. (SCHWARZ and DIBOLD, 1932; VLADIMIROVA, 1938, 1954*a*,*b*; RICHTER and DAWSON, 1948; BUDANOVA, 1950; TORDA, 1953*a*, *b*; BENITEZ, PSCHEIDT and STONE 1954; ROZENGART, MASLOVA and PANYUKOV, 1956). This work has been reviewed by WEIL-MALHERBE, (1950,1952), VLADIMIROV (1954) and VRBA (1956*b*). These results suggest that the ammonia metabolism should be considered in any attempt to formulate an enzymochemical hypothesis of nervous function, and it appeared desirable to obtain further information about the mechanisms of ammonia formation in nervous tissue. It is known that a considerable liberation of ammonia occurs when nervous tissue, and especially brain slices, are incubated *in vitro* (WARBURG, POSENER and NEGELEIN, 1924; DICKENS and GREVILLE, 1933; KREBS, 1935; WEIL-MALHERBE, 1936; WEIL-MALHERBE and GREEN, 1955). The experiments described here using guinea pig brain cortex slices are an attempt to contribute to the further elucidation of the ammonia-forming mechanisms.

A preliminary report of this work has been published elsewhere (VRBA, FOLBERGR and KANTŮREK, 1957).

METHODS

Arrangement of experiments. Unless otherwise stated, each experiment was performed with six Warburg vessels, which each contained 4 ml of Krebs–Ringer phosphate saline, no substrate being added. Each odd-numbered vessel (i.e. vessels no. 1, 3, 5) contained, in addition 1 ml of 50% (w/v) trichloroacetic acid (TCA). The vessels and contents were weighed to the nearest 0·1 mg. Guinea pigs were killed by a blow on the neck. The brain was removed and slices were cut free-hand, touched against a glass slide to remove adhering liquid and then put immediately into the vessels. From each hemisphere three slices were usually obtained making a total of six slices from each brain;

* An abstract of this paper was reported at the Second Conference on Problems of Neurochemistry, held in Kiev, 12–16 February 1957, at the Academy of Science of the Ukraine SSR.

thin slices only were used. The slices were placed in order of cutting into the numbered vessels. The first slice from the first hemisphere of a second guinea pig was put into vessel no. 2 and the last slice from the second hemisphere into vessel no. 1, and so on for each animal. In all, six guinea pigs were used in this way for each experiment, each vessel containing six slices which thus represented a more or less uniform sample of the whole batch of slices. After adding the slices the vessels were reweighed to give the weight of the slices (150–300 mg fresh weight). The vessels were then incubated in the Warburg apparatus with shaking in air at 37°C for 4 or 6 hr according to the experiment.

The whole operation from the time when the first slice was put into the first Warburg vessel until the vessels were incubated lasted about 40 min. During this time the vessels were kept on the bench at room temperature. The ability of the slices to form ammonia on incubation was apparently not impaired by the delay. The method was therefore considered to be satisfactory for the study of the problem under consideration.

After incubation, 1 ml of 50% TCA (w/v) was added to the even-numbered vessels. The contents of the vessels were then transferred to centrifuge tubes, the small pieces of tissue adhering to the walls of the vessels being quantitatively transferred to the tubes with a steel spatula. The tissue was broken up with a spatula and the tubes were centrifuged for 4–5 min at about 800 G. The supernatant fluid was then decanted into a graduated test-tube. The Warburg vessel was thoroughly rinsed with 2 ml of 10% TCA, a spatula being used to suspend any adhering tissue. The washings were transferred to the tissue debris remaining in the centrifuge tube, and after mixing and again centrifuging, the supernatant fluid was added to the first extract in the graduated test tube. The Warburg vessel was then rinsed with another 2 ml of 10% TCA and the whole operation repeated, as described above. The combined supernatant fluids were made up to 12 ml with 10% TCA. Aliquots (2 ml) were taken for the determination of free ammonia. The tissue debris was stored in a refrigerator at 0°C for further analysis.

In experiments where the effect of K^+ on the rate of ammonia formation was measured, a somewhat modified procedure was adopted.

Suspension media. The following seven media were used:

(1) Basal medium: A Krebs–Ringer phosphate saline of the following composition was used: 100 ml 0·9% (w/v) NaCl, 4 ml 1·15% KCl, 3 ml 0·11 M-$CaCl_2$, 1 ml 2·11% KH_2PO_4, 1 ml 3·82% $MgSO_4 \cdot 7H_2O$, 3 ml 1·3% $NaHCO_3$ and 11 ml 0·1 M-Na_2HPO_4 to which 20 ml per litre N-HCl had been added. The medium thus contained 146·8 mM-Na^+ and 6·25 mM-K^+ and had a pH of 7·4.

(2) Potassium-free medium: similar to the basal medium, but K salts were replaced by Na salts.

(3) 50% of NaCl in the basal medium replaced by KCl.

(4) NaCl omitted from medium and replaced by KCl.

(5) Sodium-free medium; Na salts were replaced by K salts.

(6) Medium rendered hypertonic by the addition of NaCl.

(7) Medium rendered hypertonic by the addition of KCl.

The estimation of ammonia. Ammonia was estimated by diffusion in Conway units, the centre compartment of which contained 1 ml 2% (w/v) boric acid solution containing MA and ZUAZAGA'S indicator (SOBEL, MAYER and GOTTFRIED, 1944). Appropriate quantities of the solutions to be estimated were placed in the outer compartment and made alkaline with saturated K_2CO_3 in the case of extracts made in trichloroacetic acid or acid hydrolysates (as in the determination of amide nitrogen) or with 60% KOH in the case of acidic digests. After 24 hr at room temperature (18–24°C) ammonia was titrated with 0·002 N-HCl. Solutions of ammonium sulphate carried through the appropriate procedure were included as standards with each set of determinations. All estimations were performed in triplicate. Since the amount of free glutamine in brain slices is very small (WEIL-MALHERBE and GREEN, 1955), no corrections were made for its possible degradation during the determination of free ammonia in extracts made in trichloroacetic acid (RICHTER and DAWSON, 1948).

Estimation of non-protein nitrogen (NPN). A 1 ml sample of the supernatant fluid was mixed in a test tube with 2 ml of a digestion mixture, prepared according to SOBEL *et al.* (1944), and kept in an electric oven at 280–300°C until decolorization occurred. At the end of the digestion 1–2 drops of 30% H_2O_2 were added. The mixture was then made up to 10 ml in a graduated test tube with water, and ammonia estimated in Conway dishes on a 2 ml sample.

Estimation of protein-bound amide nitrogen. The TCA-insoluble residue was transferred to a porcelain dish and the remainder of the tissue washed out from the centrifuge tube with two 1 ml

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portions of 20% HCl (w/v). The tissue was homogenized by pounding with 0·5 g quartz sand. The mixture was rinsed into a flask with 18 ml of 20% HCl (w/v) and heated in a boiling water bath for three hours. Under these conditions the tissue dissolves and quantitative splitting off of the ammonia from protein-bound amide bonds occurs (VRBA, 1957).

The hydrolysate was evaporated *in vacuo*, the temperature not exceeding 60°C, and the residue extracted successively with three 20 ml portions of water, the sand being separated each time by centrifugation. The combined aqueous extracts were evaporated *in vacuo* to dryness, and the dry residue dissolved in 12 ml water. Aliquots (2 ml) were taken for the determination of ammonia arising from protein-bound amide nitrogen.

Estimation of TCA–insoluble nitrogen. Portions (1 ml) of the tissue hydrolysate prepared as described above were digested with 2 ml of the digestion mixture of SOBEL *et al.* (1944), as described for NPN. After digestion, the mixture was made up to 10 ml and ammonia was estimated in 1 ml samples.

Estimation of 'lipid nitrogen.' Two parts of the same batch of slices, one part deproteinized with TCA before incubation, the other part after 4 hr incubation, were extracted with TCA as in the previous experiments. After extraction with TCA, 8 ml acetone was added to the tissue in the centrifuge tube, the tube was then stoppered with a ground glass joint, and after thorough shaking for 20 min on a shaker, the suspension was centrifuged and the acetone decanted. A similar extraction with 5 ml of an ethanol–ethyl ether (3 : 1 v/v) mixture was carried out, the tissue always remaining in the same centrifuge tube, followed by extraction with 5 ml of a chloroform–methanol mixture (2 : 1 v/v). Finally, the tissue residue was extracted with 5 ml of ether. The tissue debris was then discarded. The combined extracts were made up to 25 ml with methanol, and an aliquot of 10 ml evaporated to dryness on a water bath in a Kjeldahl flask. To the dry residue 2 ml of the mixture of SOBEL *et al.* (1944) was added and this was left in an electric oven at 280–300°C for three days. A further 2 ml of the digestion mixture was added and heating continued until considerable decolorization occurred; then 1 ml of 9 N-H_2SO_4 saturated with HgO was added and heating in the electric oven continued until full decolorization was observed. After this 1–2 drops of 30% H_2O_2 was added, the mixture was made up with water to 20 ml in a graduated test tube and ammonia estimated in 1 ml samples.

The lipid fraction prepared as above described, contained a considerable amount of protein, probably derived from proteolipids. A fairly complete spectrum of amino acids was found on paper chromatograms of the acid hydrolysate of the 'lipid fraction.'

Statistical evaluation of the results. Since there is considerable variation in the nitrogen content of different cortical slices, the method of arranging that each vessel contained 6 slices, one from each brain, was adopted. Comparisons were made between the individual vessels of the pairs, 1 and 2, 3 and 4, 5 and 6, on the assumption that the vessel in each pair which contained trichloroacetic acid, would act as a control for the other vessel of the pair. The use of the *t*-test is permissible under these conditions.

RESULTS

As may be seen in Table 1 (i), during incubation of the slices, appreciable ammonia formation occurred. At the same time the protein-bound amide nitrogen showed a tendency to decrease. In 11 out of 15 experiments it was observed that the concentration of protein-bound amide nitrogen was lower in incubated slices, than in the control slices, and the decrease was statistically significant ($P < 0{\cdot}02$). But whereas the mean decrease in protein-bound amide nitrogen was $-3{\cdot}8$ μmoles N, the free ammonia increased by $+16{\cdot}9$ μmoles/g. Thus only about one quarter of the ammonia formed could be accounted for by the splitting of the amide groups of the proteins. It should be mentioned that in the control slices a small amount of ammonia was formed by the non-enzymatic splitting of protein-bound amide bonds in the presence of TCA. But this was too small to affect the significance of the enzymatic splitting of protein-bound amide bonds. It is assumed that the ammonia is liberated by a direct splitting of the protein-bound amide bonds, and not as a result of the splitting off of glutamine by proteolysis, since it was found that free glutamine does not accumulate in the medium under these conditions (WEIL-MALHERBE and GREEN, 1955).

TABLE 1.—CHANGES OF AMMONIA NITROGEN, NON-PROTEIN N, PROTEIN-BOUND AMIDE N AND TCA-INSOLUBLE N IN GUINEA PIG BRAIN CORTEX SLICES INCUBATED WITHOUT ADDED SUBSTRATE. Slices incubated in Krebs–Ringer phosphate saline. Results expressed in μmoles N/g fresh weight of slices.

	Initial	Final	Mean difference	Probability	t
(i) *Slices incubated for* 4 *hr*					
(a) NH_3—N	*(17)** 6·9 (4·5–9·7)†	*(17)* 23·8 (14·2–31·8)	+16·9 ± 0·7‡	$P < 0.01$	24·1
(b) NPN	*(17)* 165·9 (139·0–242·5)	*(17)* 199·1 (132·0–273·2)	+33·2 ± 6·2	$P < 0.01$	5·35
(c) NPN corrected (NPN minus ammonia N)	*(17)* 159·1 (130·6–234·1)	*(17)* 174·5 (109·7–241·4)	+15·4 ± 5·8	$P < 0.02$	2·65
(d) Protein-bound amide N	*(15)* 83·3 (73·0–106·2)	*(15)* 79·5 (74·3–101·0)	−3·8 ± 1·5	$P < 0.02$	2·53
(e) TCA-insoluble N	*(14)* 1015 (905–1143)	*(14)* 968 (895–1092)	−47·1 ± 32·0	$P = 0.2 - 0.1$	1·47
(ii) *Slices incubated for* 6 *hr*					
(a) NH_3—N	*(13)** 5·4 (3·6–7·3)†	*(13)* 25·5 (20·1–29·8)	+20·1 ± 0·8‡	$P < 0.01$	25·1
(b) NPN	*(13)* 203·6 (139–313)	*(13)* 253·3 (197–317)	+49·7 ± 15·3	$P < 0.01$	3·25
(c) NPN corrected (NPN minus ammonia N)	*(13)* 198·1 (134–306	*(13)* 227·9 (170–290)	+29·8 ± 14·3	$P < 0.05$	2·08
(d) Protein-bound amide N	*(11)* 81·8 (70·7–92·8)	*(11)* 76·2 (66·0–85·5)	−5·6 ± 2·3	$P < 0.05$	2·44
(e) TCA-insoluble N	*(5)* 1000 (891–1081)	*(5)* 943 (792–1020)	−56·8 ± 45·7	$P = 0.3 - 0.2$	1·24

* Italicized figures in parentheses indicate the number of experiments.
† Figures in parentheses indicate the maximum range of observed values.
‡ Mean error of mean of average difference.

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The apparent decrease in the TCA-insoluble nitrogen observed in incubated slices, was not statistically significant. Proteolytic processes were thus relatively slow under these conditions. In agreement with this is the observation that the mean increase in non-protein nitrogen was +15·4 μmoles N, i.e. only about 1·5% of the total TCA-insoluble nitrogen. These facts indicate that the ammonia formation, during the incubation of guinea pig brain cortex slices is not simply a consequence of proteolytic processes.

TABLE 2.—CHANGES OF 'LIPID NITROGEN' IN GUINEA PIG BRAIN CORTEX SLICES DURING 4 HR INCUBATION IN KREBS–RINGER PHOSPHATE SALINE WITHOUT ADDED SUBSTRATE.
Results expressed in μmoles 'lipid N'/g fresh weight of tissue.

No. of experimental series	No. of vessel	1 ml of 50% TCA added before introduction of slices into vessels	1 ml of 50% TCA added after 4 hr incubation of slices	Difference
		μmoles 'lipid N'	μmoles 'lipid N'	μmoles 'lipid N'
1	1 and 2*	50†	99	+49
	3 and 4	54	95	+41
	5 and 6	60	126	+66
2	7 and 8	62	88	+26
	9 and 10	52	80	+28
	11 and 12	55	97	+42
3	1 and 2	58	88	+30
	3 and 4	43	84	+41
	5 and 6	43	65	+22
Means		53·0	91·3	+38·3 ± 4·6‡

* Into the odd-numbered vessels TCA added before introduction of the slices into vessels; into the even-numbered vessels TCA added after 4 hr incubation of both vessels. For further details see Methods.
† All figures rounded to the next whole number.
‡ $\sqrt{\Sigma\Delta^2/n(n-1)}$

In another experimental series we studied the ammonia formation in brain cortex slices incubated for six hours. The results summarized in Table 1 (ii) confirm the previous experiments and illustrate the reproducibility of the results provided that a sufficient number of experiments are performed.

Lipid fractions. In further experiments we observed that the amount of nitrogen-containing substances, extractable with organic solvents, increases significantly on incubation of the slices, as may be seen from experimental results recorded in Table 2.

After four hours' incubation at 37°C in Krebs–Ringer phosphate-saline in the absence of substrate brain slices disintegrate considerably, while the control slices, incubated simultaneously in the presence of 10% TCA, maintain their macroscopic integrity. It was therefore necessary to check whether the increased amount of lipid

nitrogen was an artefact due to the disintegrated slices being better extracted with organic solvents than the control slices. We therefore repeated the experiments reported in Table 2, with a small modification. The control slices (showing better preserved integrity) were homogenized before the extraction with organic solvents by grinding with 0·5 g quartz sand in a porcelain dish. A significant increase of lipid nitrogen in slices after incubation ($+41{\cdot}2 \pm 4{\cdot}2$; $P < 0{\cdot}001$) was again observed.

The effect of cations on ammonia formation. For the understanding of the mechanism of ammonia formation it may be important to note that although the

TABLE 3.—EFFECT OF INCREASED K^+ CONCENTRATIONS (AND DECREASED Na^+ CONCENTRATIONS) ON THE RATE OF AMMONIA AND NON-PROTEIN-N FORMATION IN GUINEA PIG BRAIN CORTEX SLICES

Flask		(a)	(b)	(c)	(d)	(e)	(f)
Incubation		4 hr	4 hr	4 hr	4 hr	4 hr	0
Medium		All K^+ replaced with Na^+	Usual Krebs–Ringer phosphate	50% NaCl replaced with KCl	100% NaCl replaced with KCl	All Na^+ replaced with K^+	Reaction stopped at zero time
Na^+ in medium (initial)		153·1 mM	146·8 mM	84·2 mM	21·6 mM	0	
K^+ in medium (initial)		0	6·25 mM	68·9 mM	131·5 mM	153·1 mM	
Free ammonia	μmoles NH_3—N/g tissue	*(8)** 27·7 (24·4–32·7)†	*(8)* 25·0 (21·3–29·0)	*(8)* 20·7 (18·1–23·8)	*(8)* 18·3 (15·1–21·2)	*(2)* 15·6 (15·9–15·2)	*(8)* 7·5 (5·1–11·2)
Non-protein nitrogen	μmoles NPN/g tissue (free NH_3 not included)	215·6 (128–287)	225·6 (197–285)	276·5 (232–327)	265·5 (215–332)	248·4 (242–255)	221·8 (183–278)

* Italicized figures in parentheses indicate the number of experiments.
† Figures in parentheses indicate the maximum range of observed values.

splitting of protein-bound amide bonds is not influenced by changes in the K^+ concentration of the medium, the greater part of the ammonia formation shows a marked dependence on the ionic composition of the medium. This was observed in experiments with brain slices suspended in different media.

Brain cortex slices from six guinea pigs were divided between 5 Warburg vessels, each containing 4 ml of medium. Vessel 1 contained 4 ml of a potassium-free Krebs–Ringer phosphate saline; vessel 2 contained the basal medium; vessels 3 and 4 contained isotonic saline with increasing concentrations of K^+ (Na^+ replaced with K^+). Four experimental series were performed in this arrangement; in two other series a fifth vessel was included, containing 4 ml of sodium-free medium. In each experimental series a control vessel was included, containing 4 ml basal medium, slices and 1 ml of 50% (w/v) TCA. After incubation for 4 hr, 1 ml of 50% TCA was added. Free ammonia and non-protein nitrogen were estimated in all vessels, as described.

Representative experimental results recorded in Table 3 show, that the greater the extracellular concentration of K^+, the slower the rate of ammonia formation. Conversely diminution of the K^+ concentration in the medium increases the rate of ammonia accumulation. We have observed that an increase in the concentration of K^+ in the medium does not decrease the accumulation of non-protein nitrogen; rather the contrary was observed in some media.

Experiments were published previously showing that $Ca^{2(+)}$ and K^+ show an antagonistic effect on the metabolism of brain cortex slices. We were not able to

demonstrate such antagonism in the case of ammonia formation in guinea pig brain cortex slices, incubated in absence of substrate. The omission of $Ca^{2(+)}$ from the medium caused only a slight decrease in the amount of ammonia formed, which was not statistically significant. On omission of $Mg^{2(+)}$, no effect was observed on ammonia formation.

In other experiments we observed that increased K^+ concentration in the medium had no apparent effect on the rate of splitting of protein-bound amide bonds; K^+ ions thus exert their effect on another process of ammonia formation.

TABLE 4.—AMMONIA FORMATION IN BRAIN CORTEX SLICES, INCUBATED IN KREBS–RINGER PHOSPHATE SALINE, RENDERED HYPERTONIC BY ADDITION OF NaCl OR KCl (NO SUBSTRATE ADDED)

Incubation (hours)	4	4	4	0
Medium	Standard Krebs–Ringer phosphate saline	Medium rendered hypertonic by addition of 125·2 mmoles NaCl/l	Medium rendered hypertonic by addition of 125·2 mmoles KCl/l	Reaction stopped at zero time with TCA
Na^+ in medium (initial)	146·8 mM	272·0 mM	146·8 mM	
K^+ in medium (initial)	6·25 mM	6·25 mM	131·4 mM	
Osmolarity of medium (initial)	309·4 mM	559·8 mM	559·8 mM	
μmoles NH_3/g wet tissue	*(6)** 24·8 (22·4–27·9)†	*(6)* 21·7 (19·0–25·1)	*(6)* 18·4 (17·4–19·1)	*(6)* 5·7 (3·7–7·9)

* Italicized figures in parentheses indicate the number of experiments.
† Figures in parentheses indicate the maximum range of observed values.

Table 4 gives the results of experiments showing the effect of K^+ on ammonia formation in guinea pig brain cortex slices, incubated in hypertonic solutions. In previous experiments the concentration of K^+ in the media was increased at the expense of Na^+ and it was not certain whether the observed inhibition of ammonia formation was a consequence of the increase in K^+ or of the decrease in Na^+ concentration. Therefore in the experiments shown in Table 4 the ammonia formation was studied in slices incubated in saline rendered hypertonic by the addition of KCl. Ammonia formation in slices incubated in the usual saline, amounted to $24{\cdot}8 \pm 0{\cdot}89$ μmoles/g, while in the hypertonic saline it was only $18{\cdot}4 \pm 0{\cdot}3$ μmoles/g; the difference is statistically significant. In parallel experiments the tonicity of the saline was increased by the addition of an equimolar amount of NaCl, as with KCl previously. In these experiments only a slight decrease of ammonia formation was observed.

These experiments indicate that the inhibition of ammonia formation in guinea pig brain cortex is a consequence of increased K^+ concentration in the medium and not of decreased concentration of Na^+.

DISCUSSION

(A) The experiments of WEIL-MALHERBE and GREEN (1955) have shown that the ammonia formation in brain slices *in vitro* is produced by various systems, of which one is presumed to be autolytic, while another depends on the structural and functional integrity of the cell.

Our results also indicate that ammonia formation in brain cortex slices is a complex process. A part of the ammonia is derived from acid-labile tissue-bound ammonia (protein-bound amide bonds), but the source of the bulk of the ammonia remains unknown. WEIL-MALHERBE and GREEN (1955) suggest that ammonia formation in brain slices is intimately linked with proteolysis, but our experiments indicate that ammonia formation cannot be considered simply as a consequence of ordinary proteolytic processes.

Our previous experiments on the nitrogen metabolism of the brain *in vivo* led us to suggest as a working hypothesis that there occurs in the brain, as a part of a functional system, a cyclic process of catabolism and anabolism of the protein fraction. The catabolic processes are characterized by deamidation and further degradation of the protein fraction, leading to the formation of ammonia. The anabolic processes are linked with the catabolic processes through the system 'free glutamic acid: free glutamine' (VRBA, 1955*a*,*b*, 1956*a*,*b*).

It may be seen in Tables 1 and 2 that in the absence of added substrate, ammonia formation occurs in brain cortex slices and at the same time, deamidation and further degradation of the TCA-insoluble protein fraction may be observed. The process of deamidation of brain proteins is shown by a decrease of protein-bound amide bonds in the slices; the process of further degradation of the TCA insoluble fraction is shown by the small, but significant increase of NPN in the medium during the incubation of brain-cortex slices. The results suggest that the catabolism of high molecular nitrogenous substances in brain slices is similar to the processes in the brain which we have previously observed *in vivo*.

The free ammonia formed in the brain *in vivo* is removed by glutamic acid and glutamine is formed (VRBA, 1955*a*, 1956*a*). This reaction has previously been shown to occur in brain slices incubated in the presence of glucose (KREBS, 1935; WEIL-MALHERBE, 1936; ÁCS, BALÁZS and STRAUB, 1953; WEIL-MALHERBE and GREEN, 1955).

(B) The work of WEIL-MALHERBE and GREEN (1955) established that none of the previously known deaminating enzymes in the brain can account for the ammonia formation, although it is probable that a small part of the ammonia formed in brain preparations may come from the action of glutamic acid dehydrogenases or adenylic deaminase (TSUKADA and TAKAGAKI, 1954; TAKAGAKI, 1955; KOMETIANI, 1954). WEIL-MALHERBE (1950) suggested the possibility that the lipids may participate in ammonia formation. We tried to find experimental support for this view in the work presented here, but the results were inconclusive.

It is generally accepted that increased functional activity of nervous tissue is associated with a loss of intracellular potassium (FENN, 1937; HODGKIN, 1951; KEYNES and LEWIS, 1955). This holds good not only for peripheral nerves, but also for the central nervous system (COLFER and ESSEX, 1947; HOAGLAND and STONE, 1948). The same physiological processes are accompanied by ammonia formation, as stated above. These facts suggest that the ammonia formation in nervous tissue and

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the diffusion of potassium out of the cells might be related. Both processes occur in brain cortex slices *in vitro*: Brain cortex slices, incubated in the absence of added substrate, lose their intracellular potassium (DIXON, 1949; TERNER, EGGLESTON and KREBS, 1950) while at the same time ammonia is liberated. The interrelation between ammonia formation and potassium ions was therefore studied.

It is difficult to explain the fact that potassium ions inhibit ammonia formation in brain slices. If the ammonia formation and potassium efflux are in some way interrelated, an increase in the potassium concentration in the surrounding medium might be expected to lead to a decrease in the diffusion of intracellular potassium into the medium, and also to a decrease in the rate of ammonia accumulation. The results of our experiments seem to agree with this assumption. WARBURG *et al.* (1924) previously showed that there is a relation between the ability of a tissue to form ammonia and its glycolytic capacity. Since potassium ions influence ammonia formation in brain slices and also exert an effect on glycolysis, a relation between both processes cannot, at present, be excluded.

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SUMMARY

(1) During incubation of guinea pig brain cortex slices for 4 or 6 hr at 37°C in Krebs–Ringer phosphate saline in absence of added substrate, considerable ammonia formation occurs. About a quarter of this ammonia may be accounted for by the splitting of protein-bound amide bonds.

(2) The increase of non-protein nitrogen under these conditions does not exceed 1·5–3 per cent of the total TCA-insoluble nitrogen of the slices. This suggests that proteolysis is very slow although a relatively large formation of ammonia occurs. These experiments give evidence for the view that most of the ammonia formation during the incubation of guinea pig brain slices is not attributable to usual proteolytic processes.

(3) The ammonia formation from brain slices is accompanied by an increase in the lipid nitrogen of the slices. This process is shown by a shift of nitrogen-containing substances from the 'protein fraction' into the lipid fraction.

(4) An increase in K^+ concentration in the medium decreases the rate of ammonia formation in brain slices; a decrease of K^+ concentration increases the rate of ammonia accumulation.

Acknowledgements—We are grateful to Dr. DEREK RICHTER for help in preparation of the manuscript. We acknowledge, with appreciation, valuable suggestions made by the editorial referee. We are indebted to Mrs. ROSA EHRMANN for careful technical assistance.

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Journal of Neurochemistry, 1958, Vol. 2, pp. 197–200. Pergamon Press Ltd., London

RATE OF COMPULSIVE CIRCLING IN RELATION TO ACCUMULATION OF CEREBRAL ACETYLCHOLINE*

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(*Received* 15 *August* 1957)

THE phenomenon of compulsive circling or forced turning in animals can be produced experimentally in a number of ways. By stimulation or ablation of specific brain parts (KENNARD and ECTORS, 1938; METTLER, 1942; WHITE and HIMWICH, 1956; etc.) as well as the injection of anticholinesterases either directly into the vascular supply leading to the brain (FREEDMAN and HIMWICH, 1949; ESSIG, HAMPSON and HIMWICH, 1953; DIAMANT, 1954; APRISON, NATHAN and HIMWICH, 1954*a*, 1954*b*; HARWOOD, 1954; NATHAN and APRISON, 1955) or into specific cerebral areas (WHITE, 1956), circus movements have been observed in animals by many experimenters. One method, the unilateral intracarotid injection of di-*iso*propyl fluorophosphate (DFP), has been used extensively in this laboratory and is excellent for the study of the correlation of certain biochemical constituents in the brain with the observed behaviour in the intact animal. In several such studies, the cholinesterase (ChE) activity (APRISON *et al.*, 1954*a*, 1956; NATHAN and APRISON, 1955) and the acetylcholine (ACh) content (APRISON and NATHAN 1956, 1957*b*) were measured in several discrete bilateral areas of the brain. This work implicated the cerebral cortex as the primary site at which a cholinergic mechanism is involved during the production of compulsive circling in rabbits. Furthermore, the asymmetrically accumulated ACh was thought to be the agent producing stimulation of one side of the cerebral cortex.

The purpose of this paper is to present data to show that (a) there is a direct correlation of the rate of compulsive circling with the asymmetric accumulation of ACh in the right and left cortices of rabbits exhibiting such movements and (b) that this is true irrespective of the direction of the forced turning.

EXPERIMENTAL

The adversive syndrome (compulsive circling to the left; 'lefter') and the reversed adversive syndrome (compulsive circling to the right; 'righter') was produced in albino rabbits weighing between 2 and 2·5 kg as described in an earlier publication (APRISON *et al.*, 1954*a*, 1954*b*). The free ACh was determined by a method recently reported (APRISON and NATHAN 1957a).

RESULTS

The data for the rabbits exhibiting compulsive circling as a result of a unilateral intracarotid (right) injection of 0·1 mg/kg DFP are presented in Fig. 1. On the ordinate axes the unit is given in μg/g of ACh. In a given animal the value for the free ACh

* A preliminary report was presented to the Section of Neurochemistry, American Academy of Neurology, Boston, Massachusetts on April 26, 1957.

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content in the left cortex is subtracted from that found for the right cortex. In 'lefters,' the right cortex contained the greater concentration of ACh and consequently the difference between the two sides of the brain results in a positive value. On the other hand, in the case of the 'righters,' the left cortex had a higher ACh content than the right and the difference in these two numbers was negative. The unit on the abscissa is 'turns per minute' measured 20 min after the injection of 0·1 mg/kg of DFP.

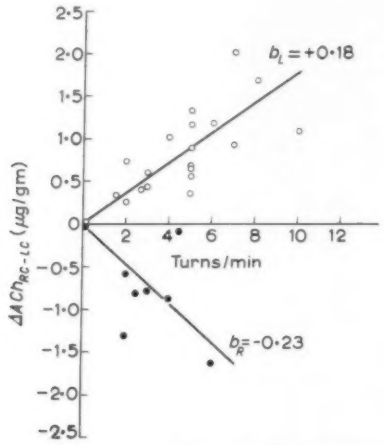

FIG. 1.—The relationship between the asymmetric accumulation of ACh in the cerebral cortices of rabbits and the rate of compulsive turns they make after the injection of 0·1 mg/kg of DFP into the right common carotid artery.

A total of 42 rabbits was used in this study. Sixteen animals were controls. These rabbits did not exhibit any turning movements (zero turns per min) but displayed a normal mode of progression after the injection of 0·1 ml/kg water. These data are represented in Fig. 1 as several points about the zero turns per min point. The mean of the difference data between the right and the left cortex [$\Delta ACh_{(RC-LC)}$] in this series of 16 rabbits is +0·03 μg/g. This would correspond to less than 0·1 turn per min (Fig. 1). Nineteen animals were 'lefters', while seven were 'righters'. Each of these latter animals is represented by a point in the figure.

The method of least-squares was used to determine the slope and equation of each line. Two assumptions were made: (1) from the data on normal animals it was assumed that the curve should pass through the origin, and (2) that the curve is linear in the region studied (0 to 10 t/min). The slope for the 'lefter' data was found to be +0·18 while that for the 'righters' was −0·23. Therefore equation (1) may be used to describe the data for the 'lefters' and equation (2) for the 'righters'.

$$\Delta ACh_{(RC-LC)} = +0·18R \quad (1)$$

$$\Delta ACh_{(RC-LC)} = -0·23R \quad (2)$$

where R is now the rate of turning 20 min after the injection of DFP (0·1 mg/kg) and subject to the limitation that it be less than 10 t/min. Since the constants in these two equations are very nearly the same, a more general equation to describe the relationship between the rate of turning, direction of turning, and the amount of asymmetry of

ACh in the right and left cerebral cortices of rabbits induced to exhibit circus movements as a result of the injection of 0·1 mg/kg DFP into the right common carotid artery is

$$\pm D = kR \tag{3}$$

where $k = 0{\cdot}2$, D is the difference in ACh content (μg/g) between the right and left cortex, R is defined as in equations (1) and (2) and a + (plus) sign indicates a counter-clockwise rotation or 'lefter' while a — (minus) sign indicates a clockwise turning movement or 'righter'.

Correlation between the ACh content in the caudate nuclei and the rate of turning was not found. In addition, when the ChE activities of the right and left cortices and caudate nuclei were used as a function to determine whether or not a relationship existed between these measurements and the rate of turning, none was found to exist.

DISCUSSION

Many studies have appeared in the literature on the action of ACh on the brain since SJÖSTRAND's paper (1937). MILLER *et al.* (1940) showed that after sensitizing a cat brain with eserine, application of ACh solutions could induce spiking in the brain waves. The described motor phenomena was thought to represent cortical stimulation. MOUSSATCHE (1945) also noted excitatory motor phenomena following the application of eserine or ACh to the motor cortex. In 1942, CHATFIELD and DEMPSEY reported that spikes could be evoked in the electrocorticogram by the addition of prostigmine, and that atropine not only could prevent this phenomenon but could eliminate it after it was once established. BRENNER and MERRITT (1942) showed that if ACh were applied directly to the cerebal cortex of the cat, the EEG changes were similar to those noted during *grand mal* epilepsy. BECKETT and GELLHORN (1948) cite evidence that changes in the cortical potentials which result from stimulation of the sciatic nerve are more marked after topical application of eserine plus ACh than in control experiments. MARRAZZI (1953, 1954) demonstrated that an intracarotid injection of micro amounts of ACh penetrated the cortex and enhanced the potential of the optic cortex when the optic nerve was periodically stimulated. Enhancement of the cortical response could also be elicited by anticholinesterase drugs. These effects were blocked when atropine was used. RINALDI and HIMWICH (1955), by noting changes in the EEG patterns, have also shown that intracarotid administration of ACh and DFP affect rabbit brain electrical activity. Studies such as that of DIAMANT (1954) and APRISON *et al.* (1956) have demonstrated that intracarotid injections of ACh can cause changes in movements in animals.

The data in the references cited above suggest that ACh is an important link in the chain of chemical events associated with excitation of cortical neurons. In addition, the observations of FELDBERG *et al.* (1940, 1942) as well as of BEUTNER and BARNES (1941, 1942) that ACh is an electrogenic agent further support this view. However, neurochemical and neurophysiological experiments to determine what levels of ACh *in situ* can cause stimulation or inhibition are lacking.

In the experiments referred to earlier (NATHAN and APRISON, 1955; APRISON *et al.*, 1956) a hypothesis was suggested to explain how circus movements could be produced in rabbits which had been injected with DFP via their right common carotid arteries. This hypothesis was based on the premise that stimulation on one side of the cerebral

cortex due to the asymmetric accumulation of ACh may be the primary factor in causing the compulsive turning. The data of this paper further substantiate this hypothesis because a relationship was found to exist between the number of turns the rabbit makes and the amount of ACh asymmetry in its cerebral cortices.

SUMMARY

A direct correlation was found between the rate of compulsive circling and the asymmetric accumulation of ACh in the right and left cortices of rabbits. This was true irrespective of the direction of the circus movements. Such a behavioural correlation was not observed in relation to the asymmetric increase of ACh in the caudate nuclei.

Acknowledgement—The author wishes to thank Dr. HAROLD E. HIMWICH for his sustained interest in this work.

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VOL.
2
1957/5

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THE BIOLOGICAL ESTIMATION OF 5-HYDROXYTRYPTAMINE (ENTERAMINE) IN THE PRESENCE OF SUBSTANCE P

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(*Received* 29 *July* 1957)

VOL. 2 7/58

SINCE 1940, a satisfactory method for the quantitative estimation of 5-hydroxytryptamine (5-HT) has been used, which depends on extraction of 5-HT from tissues with acetone and subsequent assay by the responses evoked in the isolated uterus of the oestrus rat (ERSPAMER, 1940, 1954; GADDUM and HAMEED, 1954; AMIN, CRAWFORD and GADDUM, 1954). This biological preparation is, however, rather sensitive to substance P of EULER and GADDUM (GADDUM *et al.*, 1955), which is present in acetone extracts of gastro-intestinal and nervous tissues and may therefore interfere with 5-HT estimations.

To prevent substance P passing into the acetone extracts, AMIN *et al.* (1954) recommend an extraction with 20 parts of acetone to 1 part of tissue. A number of investigations have been carried out employing this technique (AMIN *et al.*, 1954; GADDUM and PAASONEN, 1955; GARVEN 1956; GADDUM and GIARMAN, 1956). Our own control experiments (CORREALE, 1956*a*), confirmed by BENDITT (1957) and by BOGDANSKI, WEISSBACH and UDENFRIEND (1957), suggest that such treatment hinders the complete extraction of 5-HT.

An attempt has been made to separate 5-HT not only from substance P, but from all interfering tissue substances, by means of paper chromatography and elution of 5-HT from the appropriate strip (CORREALE, 1956*a*). Since this technique involves considerable loss of 5-HT, it was necessary in each experiment to estimate the actual 5-HT content of the acetone extracts by calculating from the recovery of known amounts of 5-HT added. This complicates the procedure and makes its routine use impracticable.

Of limited value, also, are the 5-HT antagonists (dibenamine, lysergic acid diethylamide, bromo-lysergic acid diethylamide). It has not yet been shown incontestably, even when they completely block the utero-stimulant effect on the sample assayed, to what extent the stimulation can be attributed to 5-HT: and when the uterine blockade is incomplete, it is even more difficult to estimate the 5-HT content of the extract. The potentiating or inhibitory action, which may operate between the substances, must also be taken into consideration (ERSPAMER, 1955*b*).

Among the various other biological test-objects used for quantitative assays of 5-HT, the atropinized rat colon (FELDBERG and TOH, 1953) seems only slightly sensitive to substance P (it appears that there may also be a qualitative difference between the effects of the two substances); the isolated mollusc heart appears to be completely insensitive (WELSH, 1953; GADDUM and PAASONEN, 1955; FÄNGE, 1955). But in spite of this, it is doubtful whether such preparations should be given the same

consideration for the qualitative and quantitative assay of 5-HT as the rat uterus, which has been the subject of a long and extensive series of experiments.

It has been shown (PERNOW, 1953, 1955) that substance P is inactivated by chymotrypsin. This enzyme was therefore used in the present experiments to avoid interference by substance P in 5-HT assays on the atropinized uterus of the oestrous rat. PARRATT and WEST (1956) have also used it recently with the same intention. Furthermore these experiments were intended to determine the actual substance P interference with 5-HT assays on the rat uterus under natural conditions.

METHODS

Experiments were carried out on the gastro-intestinal tract of the horse, dog and rat, and on the central nervous system of man, horse, dog, rat and frog: tissue extracts and bioassays of 5-HT were made using the well-known ERSPAMER technique (1940, 1954, 1955*a*), which is briefly summarized here:

The tissues, finely divided, were placed in acetone (4 ml of acetone per g of tissue) for 24 hr; the acetone was decanted and the tissues re-extracted with another 3–4 volumes of 80% acetone for a further 24 hr. The combined filtrates were kept in a refrigerator until used. The acetone was then evaporated and the residue made up to the required volume with water.

For 5-HT estimations, the uterus of the oestrous rat was used; it was suspended in an aerated 10 ml bath of Tyrode solution, containing atropine (10^{-6}), at 30–31°C. Particular attention was paid to the behaviour of the uterine tone. The standard was 5-HT creatinine sulphate (Farmitalia): the values were, however, always expressed as 5-HT base.

Substance P was extracted either by boiling the tissues for 10 min in >2 volumes of water after adjusting the pH to 4 and then repeating, or, as for 5-HT, using acetone. Substance P extracts were assayed on guinea pig ileum, suspended in a 3 ml bath of Tyrode solution at 38°C, containing atropine (10^{-6}) and mepyramine (10^{-6}), and also, occasionally, on the rabbit jejeunum (PERNOW, 1953).

Several preparations of substance P were made from horse intestine by the method of v. EULER (PERNOW, 1953) and were compared with a purified substance P sample containing 100 Euler units (E.U.)/mg, kindly provided by Dr. U. S. VON EULER.

Substance P inactivation tests were carried out as follows:

(1) Tissue extracts (1–2 g) in water	1–2 ml
(2) Chymotrypsin (Worthington) solution 1/100,000	1 ml
(3) Sodium carbonate 10%	0·1–0·25 ml
(4) Isotonic saline	to 4 ml

After checking that the pH level in the mixture was between 7·7 and 8·5, it was incubated for 2 hr at 38°C.

We often had occasion to make use of dibenamine, for the supply of which we wish to thank Smith, Kline and French Ltd. Dibenamine (5μg/10 ml) was sufficient to block the stimulant effect of 5-HT completely, while leaving unaffected the uterine response to substance P.

RESULTS

Bioassays of pure 5-HT and purified substance P in aqueous solutions

In preliminary trials it was noted that although, under the experimental conditions used, chymotrypsin alone had no effect on the uterus, it could cause the organ to react more strongly to 5-HT. This reaction was roughly proportional to the amount of chymotrypsin in the bath, and ceased when the enzyme was heated for 10 min at 90–100°C (Fig. 1). It therefore became necessary to subject each sample to this treatment after incubation. This procedure caused no loss of 5-HT activity, as was shown in a number of recovery tests.

In confirmation of previous results (ERSPAMER, 1954) the utero-stimulant action of substance P was such that 100 E.U. were equivalent to 0·1 μg of 5-HT base.

Furthermore it was ascertained that the utero-stimulant effect of the mixture of 5-HT and substance P, pre-treated with chymotrypsin, was identical to that of 5-HT alone.

5-HT had practically no effect on guinea pig ileum in the presence of atropine and

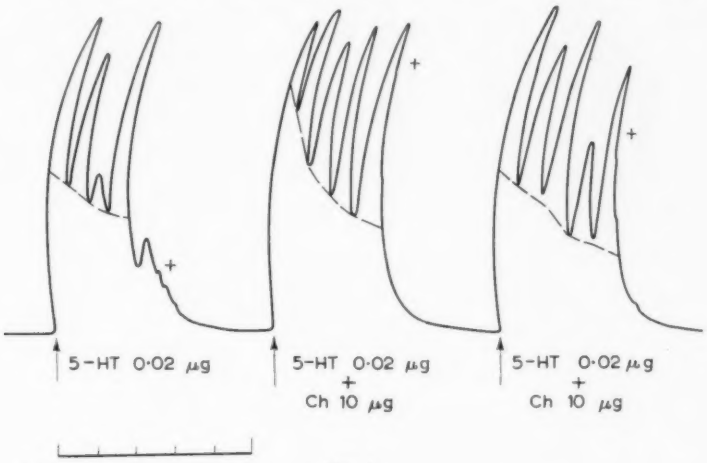

FIG. 1.

mepyramine, even when large amounts of the substance (up to 1·5 μg) were added to the nutrient bath.

Estimates of 5-HT in tissue extracts

A first series of experiments was concerned with the recovery of 5-HT added to tissue extracts in the presence of chymotrypsin. It was also desired to discover whether substance P could have any effect on the recovery. As shown in Table 1, the recoveries were always above 90 per cent with no interference by substance P.

TABLE 1.—5-HT ESTIMATES AND RECOVERIES OF ADDED 5-HT AND SUBSTANCE P IN SAMPLES INCUBATED WITH CHYMOTRYPSIN

Tissue	5-HT added (μg/g)	Substance P added (E.U./g)	5-HT content (μg/g)	Percentage recovery (%)
Rat gastro-intestinal tract	0	0	2·0	—
Rat gastro-intestinal tract	1·7	0	3·5	95
Rat gastro-intestinal tract	1·7	100	3·6	97
Rat brain	0	0	0·34	—
Rat brain	0·86	0	1·14	95
Rat brain	0·86	100	1·12	93

In Table 2 are given 5-HT equivalents of tissue extracts before and after treatment with chymotrypsin. The quantitative values found correspond approximately to those in the literature (ERSPAMER, 1954; CORREALE, 1956*a*), notable discrepancies occur only in the mid brain + diencephalon, and jejeunum of the dog. In fact, AMIN *et al.* (1954) found 0·205 μg 5-HT/g in the midbrain, 0·280 in the hypothalamus, and

0·018 in the thalamus, whereas the total value found here was 0·95 μg/g, in agreement with previous research (CORREALE, 1956*a*). The considerable discrepancy may be due either to the difference in the acetone concentrations adopted, or else to different criteria for evaluating the uterine response.

The 5-HT estimate given by ERSPAMER (1954) for dog jejeunum was 3·70 μg 5-HT/g

TABLE 2.—5-HT EQUIVALENTS (μg/g) OF ACETONE TISSUE EXTRACTS BEFORE AND AFTER TREATMENT WITH CHYMOTRYPSIN

Tissue	Before	After	Percentage difference (%)
Man midbrain	0·15	0·12	20
Horse ileum	0·35	0·32	9
Horse midbrain + diencephalon	0·40	0·28	30
Dog jejeunum	2·40	2·15	10
Dog midbrain + diencephalon	0·95	0·70	26
Rat gastro-intestinal tract	2·00	1·95	5
Rat brain	0·40	0·32	20
Frog brain	2·75	1·50	46

as against 2·4 μg/g reported here; the difference, however, does not exceed the limits of the known individual variations in normal 5-HT content.

After enzyme treatment, the values decreased by 20, 26, 30, and even by as much as 46 per cent (Fig. 2) in nervous tissue extracts; the difference in intestinal extracts was, however, in no case greater than 10 per cent.

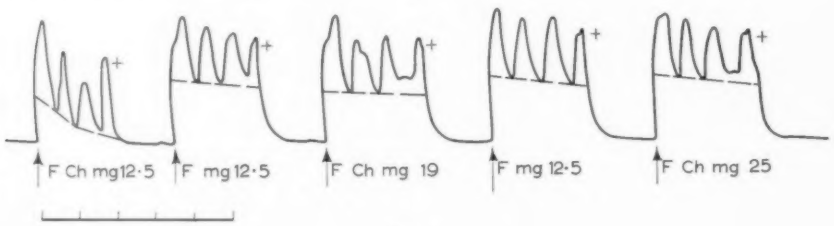

FIG. 2.

The tissue extracts not treated with chymotrypsin showed, after dibenamine had been added to the bath, a residual utero-stimulant activity which was very slight for intestinal extracts, more consistent for brain extracts and considerable for frog brain. After chymotrypsin treatment, the same extracts were completely inactive on the dibenamine-treated uterus. Only frog brain showed reduced, but still considerable, utero-stimulant action.

Estimates of substance P in the same tissues

Quantitative estimates of substance P in the same material used for the preceding experiments were carried out on the guinea pig ileum and, occasionally, on the rabbit jejeunum. The two test-objects showed no divergence in substance P values. Two types of extraction were used: with acidified water at 100°c, and with 80%

acetone. The percentage difference in the values obtained in the bio-assay of the two extracts permitted the determination of the aliquot of substance P passed into 80% acetone.

As appears from Table 3, about 30% of the substance P present in the

TABLE 3.—SUBSTANCE P ESTIMATES (E.U./g) OF AQUEOUS AND ACETONE 80 PER CENT TISSUE EXTRACTS

Tissue	Aqueous extracts	80% Acetone extracts	Percentage extracted by 80% acetone
Dog jejeunum	65	23	35
Dog midbrain + diencephalon	40	24	60
Rat gastro-intestinal tract	15	4·5	30
Rat brain	15	9	60
Frog brain	250	125	50

gastro-intestinal tract of the rat and in the jejeunum of the dog has passed into 80% acetone. This value agrees fairly well with that obtained by AMIN *et al.* (1954) for the horse intestine. For the central nervous system the aliquot was higher and varied between 50 and 60%. As already stated, the 5-HT present in the extracts cannot have influenced these estimates.

Completeness of extraction of 5-HT by different acetone concentrations

Homogenates of brain and of gastro-intestinal tract of the rat were divided into two equal aliquots, which were treated for 24 hr periods successively with the following solvents:

Aliquot a : 4 ml of acetone per g of tissue, which gives a concentration of roughly 80% with the water in the tissue; 4 vol. of 80% acetone. The residue was again extracted with 80% acetone.

Aliquot b : 20 ml of acetone per g of tissue, which gives a concentration of roughly 95% with the water in the tissue; 4 vol. of 95% acetone. The residue re-extracted with 80% acetone.

All the samples were incubated in the presence of chymotrypsin to eliminate substance P interference, and assayed on the rat uterus.

Results are shown in Table 4; 95% acetone extracted 45% of the 5-HT extracted by 80% acetone from rat gastro-intestinal tract, and 29·4% of that from the brain. The subsequent 80% acetone treatment served to extract only a part of the tissue 5-HT retained after extraction with 95% acetone. This part has been valued as a percentage (letting the 5-HT extracted by 80% acetone = 100) corresponding to 25% for the gastro-intestinal tract, and 38·2% for the brain.

DISCUSSION

It has proved possible to eliminate the stimulant action of substance P on the rat uterus, without interfering with the 5-HT bioassay, by incubating a mixture of 5-HT and substance P with chymotrypsin. It was, however, necessary to inactivate the enzyme by heating, to eliminate the potentiation of the 5-HT effect on the rat uterus by chymotrypsin. Such treatment can be profitably adopted to deal with the two systems

in which both substance P and 5-HT are normally present : the gastro-intestinal tract and the central nervous system.

In the gastro-intestinal tract, the 5-HT content is normally high in proportion to the slight utero-stimulant effect of the substance P aliquot, which passes into the acetone extracts. Therefore, particular importance is attached to the experiments

TABLE 4.—5-HT ESTIMATES OF TISSUE EXTRACTS OBTAINED WITH DIFFERENT ACETONE CONCENTRATIONS (IN PARENTHESES) AND INCUBATED WITH CHYMOTRYPSIN

Tissue	Solvent	5-HT (μg/g)	Percentage extraction
Rat gastro-intestinal tract			(2·00 μg = 100%)
Aliquot a	4 vol. acetone (80%) 4 vol. 80% acetone (80%)	2·00	
	4 vol. 80% acetone (80%)	<0·05	
Aliquot b	20 vol. acetone (95%) 4 vol. 95% acetone (95%)	0·90	45·0
	4 vol. 80% acetone (80%)	0·50 1·40	25·0 70·0
Rat brain			(0·34 μg = 100%)
Aliquot a	4 vol. acetone (80%) 4 vol. 80% acetone (80%)	0·34	
	4 vol. 80% acetone (80%)	<0·05	
Aliquot b	20 vol. acetone (95%) 4 vol 95% acetone (95%)	0·10	29·4
	4 vol. 80% acetone (80%)	0·13 0·23	38.2 67·6

carried out on the horse ileum, which is unusually poor in 5-HT and quite rich in substance P. The fact that, even in such extreme conditions, the 5-HT equivalent was reduced by only 9% after chymotrypsin treatment, is of importance in estimating the value of enzymic inactivation in general. It is concluded that, since in bio-assays it is not possible to exclude errors up to 5%, the necessity for using chymotrypsin can be limited to the special cases of 5-HT estimations on acetone extracts of gastro-intestinal tract.

In the central nervous system the conditions are the reverse. The quantities of 5-HT present are always much less than in the gastro-intestinal tract, while higher concentrations of substance P are found in just those areas of the brain which contain 5-HT. Furthermore, it has been found in these experiments that the proportion of

substance P present in 80% acetone extracts is greater. This explains the considerable decrease in the 5-HT equivalent of nervous tissue extracts, after chymotrypsin treatment. The decrease corresponds to substance P estimates found by us in the same extracts assayed on the guinea pig ileum.

Examination of the results leaves no doubt that it is necessary to eliminate the interference of substance P present in acetone extracts of nervous tissue with 5-HT assays carried out on the rat uterus. Chymotrypsin is a useful means of achieving this.

In previous researches (CORREALE 1956*a*), the qualitative identification of the 5-HT believed to be present in acetone extracts of frog brain was hindered by the simultaneous presence of a second utero-stimulant, which was subsequently found to be probably substance P (CORREALE 1956*b*). Inactivation of this substance by chymotrypsin has made it possible to trace an utero-stimulant activity in the extracts, which had been blocked to a large extent by dibenamine and which thus may, at least in part, be attributable to 5-HT.

A precise quantitative estimation is still difficult because of the presence of a third substance with an effect upon the uterus that is not modified either by chymotrypsin or by dibenamine. Recent paper chromatographic studies (CORREALE and CORTESE, unpublished observations) have given evidence of the presence of 5-HT in frog brain by application on paper of a quantity of extract corresponding to 1 g of tissue. At an approximate estimate, the quantitative chromatographic data do not differ much from those obtained by bio-assay.

The experiments with chymotrypsin have served also to confirm that the extraction of 5-HT from tissues, with 95% acetone is only partial. It seems worth while drawing attention to the difference in the proportion of 5-HT extracted from the gastro-intestinal tract and from the central nervous system, as well as to the fact that tissues pre-treated with 95% acetone retain 5-HT more tenaciously than do fresh tissues, as shown by the incomplete extraction obtained on subsequent extraction with 80% acetone.

SUMMARY

(1) Attempts were made to eliminate the possible interference by substance P in the estimation of the 5-HT content of 80% acetone extracts of tissues, assayed on the atropinized uterus of the oestrous rat. Treatment of the extracts with chymotrypsin was used with this object.

(2) Experiments were carried out on the gastro-intestinal tract of the horse, dog and rat, and on the central nervous system of a man, horse, dog, rat and frog. While discrepancies between the 5-HT equivalents of gastro-intestinal extracts examined, before and after treatment with chymotrypsin, were not above 10%, the discrepancies were more notable (up to 46%) in extracts of the central nervous system. These results agree fairly well with substance P estimates of the same extracts assayed on the guinea pig ileum.

(3) It was confirmed that 95% acetone is inadequate for a satisfactory extraction of 5-HT from tissues.

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Journal of Neurochemistry, 1958, Vol. 2, pp. 209–215. Pergamon Press Ltd., London

AMINO ACID AND PROTEIN METABOLISM OF THE BRAIN—II

THE UPTAKE OF L-LYSINE BY BRAIN AND OTHER ORGANS OF THE MOUSE AT DIFFERENT AGES

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OL.
2
7/58

(*Received* 22 *August* 1957)

INTRODUCTION

SOME time ago we reported that, after the intravenous or intraperitoneal administration of isotopic chloride or thiocyanate, equal specific activity of blood chloride and brain chloride and equal concentration of thiocyanate in the two tissues were reached more rapidly in young than in adult animals (LAJTHA, 1957).

Although it had been shown previously that glutamic acid does not enter the adult brain, recent findings indicated that this acid may be taken up in determinable amounts from the circulating blood by brains of new-born rats (SCHWERIN, BESSMAN and WAELSCH, 1950; HIMWICH and HIMWICH, 1955). These findings would support the view that for amino acids also the blood-brain barrier may not be fully operating in the immature animal.

An increased permeability of the blood-brain barrier in immature animals was first suggested by BEHNSEN (1927), who showed an increase of staining with Trypan Blue in the brains of immature rabbits. Later studies, however, with the same dye did not demonstrate penetration into the brains of embryos (GRONTOFT, 1954; GRAZER and CLEMENTE, 1957). BAKAY (1953), on the other hand, found that the brain of embryo rabbit took up larger amounts of [^{32}P]-phosphate than the adult brain. (For a discussion of these findings see WAELSCH, 1955, and LAJTHA, 1957.)

It was of interest to study the entrance of lysine into adult and immature brains since in previous experiments it had been shown that there was a rapid exchange of the free lysine of the brain with that of the circulating blood. One half of the free lysine of the mouse brain was replaced in approximately 45 min (LAJTHA, FURST, GERSTEIN and WAELSCH, 1957*a*). Since KAMIN and HANDLER (1951) demonstrated some net uptake of lysine in brain of dogs perfused for several hours with blood containing added lysine, it seemed possible that the rapid exchange rate we found was the consequence of the absence or decreased efficiency, for lysine, of a blood-brain barrier.

In this paper we should like to summarize our findings which show that, in spite of a rapid exchange, the net uptake of lysine in brain is restricted and that this restriction is more severe in adult than in immature brain.

EXPERIMENTAL

In the experiments, C_3H mice of different ages were injected intravenously and intraperitoneally with a solution containing 20 to 40 mg of L-lysine per ml, neutralized to pH 7·2; 0·01 to 0·02 ml/g body weight was administered. After a predetermined time period the animals were killed by decapitation. The organs were frozen in dry ice, weighed, and homogenized in 15 vol. 5% trichloroacetic acid (TCA) and the suspension was centrifuged. The residue was extracted twice more with 5% TCA. The lysine content of the combined TCA extracts was then determined. The blood of young mice and adults was collected in small dishes containing sodium citrate and quickly centrifuged; the plasma was then frozen, weighed, and extracted with 5% TCA. In the case of new-born animals whole blood was used.

The methods employed for the determination of free lysine have been previously described in detail (LAJTHA, FURST and WAELSCH 1957*b*). They consisted briefly of the following: TCA was removed from the extract by passing the solution through Dowex 2 (Cl-form); the resin was washed with 0·02 N-HCl. The combined effluents were brought to dryness *in vacuo* in a rotary evaporator. The residue was taken up in water, the pH adjusted to 7 with NaOH, and the lysine content determined by the microbiological method of HENDERSON and SNELL, with the modifications described previously (LAJTHA *et al.*, 1957*b*). The determination of the blood content in the organs followed with slight modifications GORDON and NURNBERGER'S method (1955, 1956; LAJTHA *et al.*, 1957*b*).

RESULTS

The results of the determination of free and protein bound lysine are shown in Table 1. The concentration of protein bound lysine is the same in various organs of

TABLE 1.—LYSINE CONTENT OF MOUSE TISSUES
(μg/g) fresh tissue ± S.D.

		Plasma	Brain	Liver	Muscle	No. of animals
Free lysine in the organ μg lysine/g fresh tissue	Adult	50 ± 8	38 ± 3	89 ± 4	129 ± 16	4 + 4 + 6 + 6 + 6
	Young	49 ± 1	51 ± 3	71 ± 5	381 ± 11	22 + 20 + 25 + 25
	Newborn	133 ± 16	81 ± 4	122 ± 10	319 ± 26	15 + 21 + 22 + 24
Protein bound lysine μg lysine/mg dry protein	Adult	85 ± 1	66 ± 3	83 ± 1	103 ± 4	4 + 4 + 6 + 6 + 6
	Young	84 ± 3	68 ± 2	84 ± 2	100 ± 3	22 + 20 + 25 + 25

young and adult animals. It varies between 7 and 10 per cent in the proteins of different organs but is fairly constant in the total proteins of the same organ from one animal to the other. The free lysine content of the organs, however, varies greatly; it is highest in new-born animals, lower in young animals, and still lower in adults. Although the free lysine content shows considerable variation in the organs of different animals of the same age group, these differences are considerably smaller than those between animals of different ages. The lysine content of tissues after the injection of varying amounts of lysine into mice is shown in Table 2. All the values are corrected for the lysine content of the blood present in the organs.

Table 3, which shows the increase in lysine content over control, was calculated from Table 2. All values are expressed as μg lysine/g wet tissue. The lysine increment

Table 2.—LYSINE CONTENT OF MOUSE ORGANS AFTER INJECTION OF L-LYSINE (μg/g fresh tissue)

μg lysine/g body weight injected	No. of animals pooled	Time interval	Plasma	Brain	Liver	Muscle	Mode of administration§
			Adult (100 day old)				
200	6 + 6	5	259	36·9	424	229	IP
200	6 + 6	10	256	38·7	517	180	IP
200	6+ 6	20	183	40·0	435	208	IP
400	5 + 5	5	830	53·4	1306	339	IV
400	4 + 4	10	324	37·3	962	284	IV
400	5 + 5	20	260	47·6	950	278	IV
800	4 + 4	5	1860	72·8	1820	474	IV
800	5 + 5	10	684	58·4	1975	372	IV
800	4 + 6	20	530	68·2	1750	463	IV
Control	4 + 4 + 6 + 6 + 6		50·0	38·0	88·6	129·4	
			Young (10 day old)				
400	8	5	718	79·6	756	744	IP
400	8	10	639	97·8	613	884	IP
400	8	20	562	118·2	895	930	IP
800	5	5	1270	76·7	1310	808	IP
800	5	10	1580	97·1	1160	1087	IP
800	5	20	1530	107·4	1420	1026	IP
Control	22 + 20 + 25 + 25		49·0	51·0	71·4	382	
			New-born (less than 24 hr old)				
			Blood				
200	16 + 15 + 15	5	347	117·7	316	360	IP
200	17	10	314	122·2	313	349	IP
200	17 + 14	20	222	120·7	492	417	IP
400	17 + 16 + 16	5	425	100·8	522	507	IP
400	13 + 15	10	355	116·0	679	607	IP
400	17	20	455	160·0	938	820	IP
800	12	5	850	140·1	892	1131	IP
800	12 + 16	10	723	125·0	740	860	IP
800	13	20	838	153·0	1570	1150	IP
Control	15 + 21 + 22 + 24		133	81·4	122	319	

§ IP intraperitoneal injection; IV intravenous injection.

in tissues is shown to depend on several factors: the plasma level of lysine, the duration of the experiment, and the age of the animal. As expected, with increasing level of plasma lysine, the concentration of lysine in muscle or liver increases in parallel fashion. The duration of the experiment does not have a clear-cut influence. The increases in liver free lysine approach and frequently surpass the plasma values, a finding in agreement with the role of the liver in removing excess free amino acids

TABLE 3.—INCREASE OF LYSINE IN MOUSE ORGANS OVER CONTROL VALUES AFTER INJECTION OF LYSINE
(μg lysine/g fresh tissue)

μg lysine/g body weight injected	No. of animals pooled	Time interval	Plasma	Brain	Liver	Muscle	Mode of administration
			Adult (100 day old)				
200	6 + 6	5	209	0	335	100	IP
200	6 + 6	10	206	0·7	428	51	IP
200	6 + 6	20	133	2·0	346	79	IP
400	5 + 5	5	780	15·4	1220	200	IV
400	4 + 4	10	274	0	873	155	IV
400	5 + 5	20	210	9·6	861	149	IV
800	4 + 4	5	1810	34·8	1730	335	IV
800	5 + 5	10	634	20·4	1890	243	IV
800	4 + 6	20	480	30·2	1660	334	IV
			Young (10 day old)				
400	8	5	669	28·6	685	362	IP
400	8	10	590	46·8	542	502	IP
400	8	20	513	67·2	824	548	IP
800	5	5	1220	25·7	1240	426	IP
800	5	10	1530	46·1	1090	705	IP
800	5	20	1480	56·4	1350	644	IP
			New-born (less than 24 hr old)				
200	16 + 15 + 15	5	214	36·3	194	41	IP
200	17	10	181	40·8	191	30	IP
200	17 + 14	20	89	39·3	370	98	IP
400	17 + 16 + 16	5	292	19·4	400	188	IP
400	13 + 15	10	222	34·6	557	288	IP
400	17	20	322	78·6	816	501	IP
800	12	5	717	58·7	770	812	IP
800	12 + 16	10	590	43·6	618	541	IP
800	13	20	705	71·6	1450	831	IP

from the circulation. Muscle shows an increase which in this set of experiments is larger in new-born than in young, the increases in both age groups being larger than that in the adult.

Compared to muscle or liver, the increases in the free lysine content of the brain in adult animals are very small. The uptake of lysine by the brain depends on the amount injected, the length of the experimental period, and the age of the animal much more markedly than that by liver or muscle. The brain of the young or new-born mouse takes up considerably larger amounts of excess lysine from the circulation than does that of the adult mouse. Actually, in the experiments in which 200 or 400 μg

of lysine/g were administered to adult mice, the increase of free lysine in the brain is questionable, while significant increases were found in the liver.

DISCUSSION

The experiments presented in the report demonstrated that the net uptake of lysine is considerably higher by the brain of new-born or young mice than by that of adult mice. The blood brain barrier of the adult animal seems to be able to prevent the net uptake of the basic amino acid if the lysine concentration of the circulating blood is only slightly elevated. Excessive increases of the lysine concentration in plasma in our experiments, or continuous infusion (KAMIN and HANDLER, 1951) result in a net uptake of the amino acid by the brain.

In the experiments in which glutamic acid was administered to rats or mice (SCHWERIN *et al.*, 1950) a fifty- to a hundred-fold increase of the plasma concentration of the dicarboxylic acid did not lead to a significant increase of the concentration of the amino acid in the brain, while a tenfold increase of glutamine in the blood sufficed for a significant rise of the amide concentration in the brain. Furthermore, the increase of the glutamic acid in the liver after its administration could be explained by equilibration of the plasma concentration with that of the extracellular fluid phase. Such an explanation can hold neither for the enormous increase of the liver concentration after the administration of the amide nor for the increase in this organ of the lysine concentration after the administration of the basic amino acid. It appears, therefore, that as to uptake by the liver and brain, glutamine and lysine behave similarly.

It is clear that almost all the lysine taken up by liver is in the intracellular phase while in muscle a considerable portion might be in the extracellular phase. Lysine which may be considered metabolically stable in experiments of short duration might give a better picture of the function of a blood-organ barrier than an amino acid such as glutamic acid which is metabolically more active (BERL, LAJTHA and WAELSCH, 1957).

It has been reported that foetal tissues contain high levels of free amino acids which are assumed to be necessary for the high synthetic activity in developing tissues (CHRISTENSEN and STREICHER, 1948). Similarly in our experiments the free lysine content of tissue was higher in immature than in adult animals. It is not known at the present what influence, if any, the decrease of the extracellular phase in development exerts on the lysine content. If part of the free lysine in the organ is present in the extracellular phase, one might expect a decrease of the lysine concentration as the extracellular phase decreases.

It was shown that in potassium-depleted animals lysine could replace part of the muscle potassium. High levels of administered basic amino acids could replace part of the potassium in muscle (ECKEL, POPE and NORRIS, 1954; JACOBELLIS, MUNTWYLER and DODGEN, 1956), and also in Ehrlich ascites cells (CHRISTENSEN, 1955). Further experiments will show whether the uptake of lysine in our experiments is accompanied by a loss of potassium and whether the replacement of potassium by lysine is more rapid or extensive in immature than in adult tissues. This is of particular interest in connection with the observation that a part of the potassium of the adult brain is in a bound form while in young animals all of the brain potassium is exchangeable (KATZMAN and LEIDERMAN, 1953).

The question may also be asked whether or not increase of lysine in any organ leads to changes in the concentration of other amino acids such as a gain in dicarboxylic amino acids.

Although the increase in muscle lysine parallels the levels of plasma lysine in young animals fairly well, in adults it remains below the increase of the plasma lysine levels. This finding indicates that, as for brain, net uptake of lysine by the muscle decreases with age. There are some differences, however, between muscle and brain. The decrease with age of the free lysine content in muscle of the control animals is not considerably greater than it is in brain. The amount of free lysine in muscle exceeds that of the brain considerably at all ages. Finally, the permeability barrier in brain seems to be stronger than it is in muscle—the brain at any time taking up much less lysine than does muscle, although the normal lysine concentration in muscle is considerably higher than in plasma. While the amount of lysine entering the brain of immature mice is considerably greater than it is in adults, the brain lysine concentration never approaches the plasma content, even in the new-born animals. This finding suggests that also in the new-born animal there is a blood-brain barrier for lysine, although it is less effective than in the adult animal. The rapid exchange of the free lysine of blood with that of brain is in contrast to the lack of net uptake of lysine by the brain when the blood concentration of this amino acid is only moderately increased. This observation underscores the complexity of the processes underlying the concept of the blood-brain barrier.

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SUMMARY

The lysine content of new-born, young and adult mice has been measured before and after the injection of L-lysine. If the plasma concentration of lysine was only moderately increased, the net uptake of lysine by the free amino acid pool of adult brains was not significant, while new-born brains showed a considerable uptake. The findings suggest that the blood-brain barrier is less effective for lysine in the new-borns than in adults.

Muscle showed behaviour similar to that of brain although it was less pronounced.

The significance of the finding of a restriction of net lysine uptake in spite of rapid exchange is discussed.

Acknowledgements—This work was supported by grants from the National Institute of Neurological Diseases and Blindness (Grants B-226 and B-557) of the National Institutes of Health, Public Health Service, Scottish Rites Supreme Council, and by a contract between the Office of Naval Research and the Psychiatric Institute. The author wishes to express his thanks to Professor HEINRICH WAELSCH for his encouragement and advice. Mr. ALBERT GERSTEIN's skilful help is gratefully acknowledged.

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Journal of Neurochemistry, 1958, Vol. 2, pp. 216–225. Pergamon Press Ltd., London

AMINO ACID AND PROTEIN METABOLISM OF THE BRAIN—III

INCORPORATION OF LYSINE INTO THE PROTEINS OF VARIOUS BRAIN AREAS AND THEIR CELLULAR FRACTIONS

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(*Received* 22 *August* 1957)

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RECENTLY we reported (LAJTHA, FURST and WAELSCH, 1957*a*; LAJTHA, FURST, GERSTEIN and WAELSCH, 1957*b*) the results of a study of the rejuvenation of both the free lysine and protein bound lysine of brain, liver, and muscle of mice. From the data on the flux of free lysine from the circulating blood into the brain, a half-lifetime of less than 45 min was estimated for the free lysine of this organ, while the corresponding value for free liver lysine was less than 10 min. The half-lifetime of the organ proteins was calculated from the rate of flux of lysine from the non-protein fraction into the protein. It was found that, with increasing time interval between administration of the labelled amino acid and sacrifice of the animals, increasing half-lifetimes were obtained. This finding was interpreted as indicating that in short time experiments the turnover of rapidly metabolized proteins was primarily measured, while in experiments of longer duration, the metabolic activity of the slowly metabolized proteins became prominent.

In a relatively homogeneous organ such as the liver the heterogeneity of turnover rates of organ proteins is attributable to the metabolic differences between the parenchymatous and connective tissue elements and in particular to dissimilarity amongst the proteins of the different cellular fractions, such as nuclei, mitochondria, microsomes, and the soluble portion. In the brain, the spectrum of heterogeneity may be further extended by the presence of various functional areas which differ widely not only in the characteristics of the cellular elements but also in the relative preponderance of perikarya, glial cells, and myelinated structures.

In this report, our findings on the turnover rates of the proteins of different areas of the monkey brain and of the different cell fractions in each of these areas are summarized. In the course of these studies, data on the concentration of lysine in the respective proteins and in the non-protein fractions of different areas of the central nervous system were collected. Furthermore, the rate of lysine incorporation into a relatively well-defined family of proteins—the proteolipids—was studied. The possibility that post-mortem changes distorted results obtained on incorporation of [^{14}C]-lysine into the different nitrogenous fractions was excluded by several specially designed experiments.

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EXPERIMENTAL

Incorporation of [^{14}C]*-lysine into the non-protein nitrogen fractions and proteins of different areas of the central nervous system.* Adult monkeys (*Macacca Irus*) weighing 1 to 1·5 kg were injected via the leg vein with uniformly labelled L-[^{14}C]-lysine (Nuclear-Chicago Corp.). The lysine solution contained 13 mc/m-mole 13·6 μc/kg body weight was injected in a total volume of 0·3 ml. After the experimental time the animal was killed by decapitation, the skull opened with an electric saw, the brain dissected in the cold room, and the various parts frozen in dry ice. While the dissection, which was carried out at 0–4°, was of necessity rapid, care was taken in the preparation of each area in order to preclude, as far as possible, contamination by adjacent parts. The brain parts were all frozen within 3–12 min of the decapitation of the animal.

The concentration of free and protein-bound lysine and its radioactivity were determined as described in detail elsewhere (LAJTHA *et al.*, 1957*b*). Briefly, the non-protein nitrogen fraction was obtained by extraction of the brain parts with 5% trichloroacetic acid (TCA) and subsequent removal of the TCA on Dowex 2 columns. After the removal of the TCA the solutions were brought to dryness *in vacuo;* the residues were dissolved in water and neutralized. Proteins were extracted with cold and hot TCA, with acetone, then with chloroform–methanol (2 : 1 v/v) at 50° for 10 min, followed by ethanol–ether (1 : 1 v/v) at 40° for 10 min, and finally with ether. The radioactivity of the non-protein nitrogen fractions was determined by plating the solution on aluminum planchets, while proteins were counted as powders. Corrections were made for self-absorption. The lysine concentration was determined microbiologically in the non-protein nitrogen fraction after removal of TCA, and in the proteins after hydrolysis.

Separation of proteolipids. Essentially, the procedure of FOLCH and LEES (1951) was followed. Tissues were homogenized in 20 vols of chloroform-methanol (2 : 1 v/v) mixture at 0°. In Experiment 1 the mixture was kept in contact with water for 48 hours and the fluff (proteolipids A + B) and the chloroform phase (proteolipid C) separately worked up. In Experiment 2 the chloroform–methanol mixture was first extracted several times with 5% TCA and then brought to dryness. In both experiments the proteolipids were washed twice with cold 5% TCA, kept at 90° in 5% TCA for 15 min, centrifuged, washed with cold 5% TCA, then with acetone, and twice with 5% TCA. Finally they were washed twice with acetone, twice with chloroform, and three times with ether, and were dried. Counting of the radioactivity, hydrolysis, and determination of the lysine content was done as described elsewhere (LAJTHA *et al.*, 1957*b*).

Determination of post-mortem changes. Several groups of mice were injected intravenously with radioactive lysine and decapitated after 5 or 25 min. Half of each brain was placed in dry ice and the other half in a stoppered weighing bottle. Both were weighed, and the frozen half homogenized immediately in 5% TCA, the other half after 20 min incubation at 37°. The specific activities of free and protein bound lysine were determined as described (LAJTHA *et al.*, 1957*b*). Results showed that *in vitro* incubation at 37° for 20 min, a time period in excess of that needed for dissecting the brains at 0–4°, did not significantly alter the radioactivity of the brain or liver proteins or of the respective non-protein nitrogen fractions (Table 1). The data obtained also indicated that the biosynthetic processes were terminated by the death of the animal.

Incorporation of [^{14}C]*-lysine into cellular fractions of various brain areas. Isolation of cellular fractions.* The method of injection and of removal of the brain and dissection of areas was carried out as described above. In the first series of experiments, cortical tissue alone was employed. This was rapidly dissected away from underlying parts and homogenized in 9 vols. of ice-cold 0·25 M-sucrose solution. In the second series of experiments, as noted, separate areas of the cortex, as well as other parts of the brain were dissected and homogenized in sucrose solution. In one experiment, a laminectomy was performed and the entire spinal cord removed.

After homogenization in an all-glass Potter-Elvehjem homogenizer, the suspensions were subjected to differential centrifugation in order to obtain various particulate cell fractions. In order to check the efficiency of the separation technique employed, a small aliquot of each cell fraction was prepared for examination in the electron microscope. While these examinations showed each sample to consist largely of the desired fraction, no statement as to their absolute homogeneity (particularly when derived from areas other than the cortex) can be ventured at this time. We are indebted to Dr. H. RUSKA for help with the preparation and examination of the electron micrographs.

The procedure of fractional centrifugation, which was similar to that proposed by BRODY and BAIN (1952), is summarized in Table 2. Each fraction was resuspended once in sucrose solution and

TABLE 1.—CHANGES IN RADIOACTIVITY OF PROTEIN AND NON-PROTEIN NITROGEN FRACTIONS OF BRAIN AND LIVER UPON INCUBATION

Experiment	Incubation at 37° (min)	Counts/min/g fresh tissue			
		Brain (5)		Liver (2)	
		Protein	Non-protein nitrogen	Protein	Non-protein nitrogen
A	0	301	2,177	6,325	13,765
	20	306	2,186	6,455	13,345
	Δ%	+1·7	+0·4	+2·1	+3·1
B	0	976	2,091	12,470	4,437
	20	940	2,063	13,200	4,212
	Δ%	−3·7	−1·3	+5·9	−5·1

In experiment A, the animals were sacrificed 5 min after administration of [^{14}C]-lysine, in experiment B 25 min after.

TABLE 2.—FRACTIONAL CENTRIFUGATION OF BRAIN HOMOGENATES

Fraction No.	Content	Centrifugation (× G)	Time of centrifugation (min)
1	Whole homogenate		
2	Unbroken cells, tissue fragments, occ. nuclei	800	10
3	Nuclei, occ. small tissue fragments	1,500	10
4	Mitochondria	12,000	15
5	Large microsomes	35,000	30
6	Intermediate microsomes	78,500	60
7	Small microsomes	78,500	240
8	Supernatant fluid		

The International Refrigerated and Spinco centrifuges were used.

recentrifuged at the same speed and for the same time as was used for its procurement. From the combined supernatant and wash fluids the next fraction was obtained by further centrifugation. From each fraction aliquots were removed and prepared for electron microscopy according to the method of DIANZANI and BAHR (1954).

The remaining material was homogenized in 5% TCA and the proteins prepared as described above. The proteins of the supernatant fluid (Fraction No. 8) were precipitated by the addition of 50% TCA in sufficient quantity to make the solution 5% with respect to TCA. The precipitate was then treated as were all other fractions.

Calculation of flux and half-life time. See LAJTHA *et al.* (1957*b*).

RESULTS AND DISCUSSION

Concentration of free and protein-bound lysine

The concentration of free lysine, as determined by the microbiological method, is about 20 per cent lower in the cortices than in other parts of the central nervous system (Table 3). On the other hand, the proteins of the cortices and of the cerebellum

TABLE 3.—CONCENTRATION OF FREE AND PROTEIN-BOUND LYSINE OF DIFFERENT PARTS OF BRAIN (MONKEY)

	Free lysine (μg/lysine/g fresh tissue)	Protein lysine (μg/lysine/mg dry protein)
Plasma	20	98
White matter	35	39
Cord	35	38
Medulla	35	42
Pons	35	40
Hypothalamus	34	49
Thalamus	34	54
Cerebellum	38	61
Frontal cortex	29	64
Motor cortex	26	63
Sensory cortex	29	61
Temporal cortex	28	64
Occipital cortex	27	61

contain a considerably higher concentration of lysine than the myelinated structures, hypothalamus and thalamus. The lysine concentration of the proteins of the myelinated structures may, in reality, be somewhat higher, if all the lipid material was not removed by the extraction procedure, although a more vigorous extraction of lipids was used in these experiments than previously. It is interesting to note that the cerebellum has the highest concentration of free lysine and that, in addition, its proteins belong to those with a high lysine content. The analytical data served for the determination of specific activities after the administration of [^{14}C]-lysine.

Incorporation of lysine into various brain areas

The determination of the specific activity of free lysine in different parts of the central nervous system after the administration of isotopic lysine bears out the finding reported previously (LAJTHA *et al.* 1957*a*) that lysine exchanges rapidly between blood and brain (Table 4). In the animals sacrificed from 5 min up to 45 min after the intravenous injection of the lysine solution, the free lysine of the corpus callosum and its radiation (white matter) shows the lowest specific activity, while the free lysine of the predominantly grey structures has a considerably higher specific activity. Approximately the same relationship holds true for the specific activities of the protein bound lysine of the different brain parts (Table 5). It should be noted that the proteins of the corpus callosum contain lysine with a higher specific activity than those of the spinal cord. The specific activity data were utilized for the calculation of the flux of free lysine into protein lysine and for computing the half-lifetime of

TABLE 4.—SPECIFIC ACTIVITIES OF FREE LYSINE IN DIFFERENT PARTS OF BRAIN

Duration of experiment (min)	5	10	15	30	45
	Counts/μg/free lysine/min				
Plasma	1,034	522	278	167	153
Spinal cord	137	133	110	91	97
White matter	84	83	71	93	83
Medulla pons	175	179	133	148	114
Thalamus-hypothalamus	152	163	137	127	124
Cerebellum	178	181	135	140	124
Motor cortex	280	210	187	140	124
Sensory cortex	215	241	162	155	134
Temporal cortex	211	230	206	138	126

TABLE 5.—SPECIFIC ACTIVITIES OF PROTEIN-BOUND LYSINE OF DIFFERENT PARTS OF BRAIN (MONKEY)

Duration of experiment (min)	5	10	45
	counts/μg lysine/min $\times$ 100		
Plasma	0·7	6·3	57·4
White matter	5·3	9·2	39·0
Spinal cord	4·1	4·7	15·6
Medulla	6·5	11·8	32·4
Pons	9·0	11·7	43·6
Hypothalamus	8·8	11·9	32·0
Thalamus	8·3	11·5	27·5
Cerebellum	8·3	13·6	41·5
Rest of brain	10·6	19·5	45·2
Frontal cortex	13·6	22·9	61·0
Motor cortex	13·6	22·6	59·0
Sensory cortex	12·4	22·5	65·8
Temporal cortex	13·6	19·4	43·0
Occipital cortex	12·8	25·0	

TABLE 6.—HALF-LIFETIMES OF PROTEINS IN MONKEY BRAIN AREAS

Duration of experiment (min)	5	10	45
	Half-lifetime (days)		
Cord	10·6	9·5	18·5
White	3·9	4·0	8·7
Medulla-pons	6·6	6·7	13·7
Thalamus-hypothalamus	6·1	7·8	16·8
Cerebellum	5·4	7·6	14·1
Cerebral cortex	5·5	6·7	12·7

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the proteins (Table 6). The average half-lifetime values calculated from the 45 min experiment are in agreement with those given for whole brain proteins after the administration of [^{35}S]-methionine to rats by GAITONDE and RICHTER (1956).

It is striking to note that the protein of the corpus callosum shows a half-lifetime considerably shorter than the proteins of the cortex or cerebellum. At present no definite explanation can be given for these unexpected results. In order to explore the problem further, very careful removal of the cortex by suction and of the inner and outer part of the corpus callosum and its radiations were made in collaboration with Dr. FRED A. METTLER. The protein-bound lysine of the frontal cortex removed by suction showed, in the three experiments carried out, a wide variation in specific activity relative to that of the protein-bound lysine of the white matter. The specific activities of the free lysines of the two parts of the corpus callosum were not as divergent as those found in the previous experiments. While from these experiments it may appear that the rate of turnover of the cortical proteins is higher than that of the white matter, the careful preparation of the different parts took considerably more time than the preparation of the brain parts in the previous experiments. The specific activities of the proteins of the outer and median parts of the corpus callosum showed no reproducible differences; in two experiments the proteins of the inner part had a lower specific activity than the outer part, and in one experiment the reverse was true. As pointed out above, the high rate of turnover of the proteins of the white matter is the result of the low specific activity of the free lysine of this area. Considerably lower turnover values would be obtained if the specific activity of the protein-bound lysine should be related to another compartment containing free lysine with higher specific activity such as the cortex, from which the axons of the corpus callosum and its radiation are mainly derived. This would presuppose a very rapid flow of the proteins through the axoplasm, a suggestion which is not borne out by the analyses carried out on the outer and inner part of the white matter referred to above. Specially designed experiments, to be reported shortly, in which protein synthesis was studied in the sciatic nerve of the frog and spider monkey, indicate a gradient of specific activity of protein lysine from the spinal cord distally, but cannot, at the present time, be interpreted with certainty as proof of the flow of axoplasm. Be that as it may, the flow of axoplasm in the corpus callosum would have to be extremely rapid in order to make its appearance within such a short time. It is more likely that within the white matter there are different compartments of free lysine, one which exchanges rapidly and is utilized for protein synthesis, another which exchanges slowly and consequently has a very low specific activity. The finding of a low specific activity for the free lysine may be the result of mixing of the compartments during isolation and may be taken erroneously for the basis of calculation of the turnover rates of the proteins of the white matter. In this connection, it should be remembered that HELLER and ELLIOTT (1955) have found a high respiratory rate of the corpus callosum, and the possibility exists that the proteins of the glial cells of this particular area of the brain have a very rapid turnover.

All parts of the brain analysed again show the heterogeneity of the component proteins and it can be seen that, on the one hand, the proteins of the cord, and on the other, those of the cortex, cerebellum, thalamic-hypothalamic areas and corpus callosum extend the spectrum of different turnover rates of the brain. Although it would be inviting to speculate on the basis of the data as to the relative proportions

of proteins with high and low rates of turnover in the different areas, the accuracy of the methods applied does not permit such an evaluation.

Incorporation of [^{14}C]-*lysine into cellular fractions*

The procedure for the separation of the various cellular fractions of brain tissue has been outlined in the experimental part. Although the purity of the fractions has been checked by electron microscopy, it should be reiterated that all fractions represent

TABLE 7.—LYSINE CONTENT OF PROTEIN FROM PARTICULATE FRACTIONS (μg lysine/mg protein*)

Fraction†	Cortex	Cerebellum	Thalamus-hypothalamus	Pons-medulla	Cord	White matter
2	50	43	37	41	28	33
3	55	57	49	41	24	40
4	50	49	43	42	29	44
5	58	46	44	45	27	45
6	64	50	62	65		
7	73	73	72	74	65	60

* Averages of several determinations from two separate experiments.
† See Table 2.

a composite mixture of various cellular components with more or less significant predominance of the designated fraction. The naming of the fractions as nuclei, mitochondria, and various microsomes, is, therefore, done only for simplicity's sake in order to define the centrifugal force and time of centrifugation applied. The concentration of lysine in the various cellular fractions from various brain areas is given in Table 7. The soluble proteins contain the highest concentration of the diamino acid. The different cellular fractions of the cortex were isolated and analysed

TABLE 8.—SPECIFIC ACTIVITY* OF PARTICULATE FRACTIONS OF CORTEX

Duration of experiment (min)	5	15	30
	Counts/min/μg lysine		
Cell debris and nuclei	0·09	0·19	0·76
Nuclei	0·14	—	0·65
Mitochondria	0·27	0·20	0·57
Large microsomes	0·39	0·67	1·29
Intermediate microsomes	0·54	0·86	1·36
Small microsomes		0·31	
Supernatants	0·17		

* Randomly labelled [^{14}C]-lysine, containing 0·05 μc/μg, administered at level of 0·3 μg/g body weight.

for the specific activity of the protein-bound lysine from monkeys which received [^{14}C]-lysine, and which were sacrificed 5, 15, and 30 min after the administration of the amino acid (Table 8). It is apparent that those fractions sedimented at the highest centrifugal forces and designated as various microsomal fractions showed the highest rate of incorporation of the isotopic amino acid. The same picture emerges in the fractions prepared in the same way from the different parts of the brain (Table 9). Although these fractions are mainly characterized by the centrifugal force used to sediment them in 0·25 M-sucrose solution, all 'microsomal' fractions show by far the highest rate of incorporation of [^{14}C]-lysine. Similar results have recently been reported by CLOUET and RICHTER (1957).

TABLE 9.—RELATIVE SPECIFIC ACTIVITIES OF PARTICULATE FRACTIONS FROM VARIOUS BRAIN AREAS

	Cortex	Cerebellum	Thalamus-hypothalamus	Pons medulla	Cord	White matter
Whole homogenate	0·26 = 1·0	0·18 = 1·0	0·18 = 1·0	0·13 = 1·0	0·1 = 1·0	0·19 = 1·0
Cell debris, nuclei, etc.	0·97	1·12	0·53	0·80	0·77	0·67
Mitochondria	0·82	1·21	0·80	1·04	1·07	0·86
Large microsomes	1·33	2·61	1·44	1·27	1·43	1·88
Intermediate and small microsomes	3·86	3·20	2·13	1·82	1·80	2·73
Supernatant	1·47	1·15	1·10	1·17	1·17	1·20
'Nucleoproteins'	1·78					
Supernatant from above	1·39					

Specific activity of whole homogenate = average for area = 1·0. Relative specific activity for fractions = $\frac{\text{specific activity of cell fraction}}{\text{specific activity of whole homogenate}}$.

The specific activity of the lysine of the microsomal fractions is, depending on the experimental period, up to fivefold that of the whole homogenate. An estimate of the approximate half-lifetime of these fractions would lead to a value of less than one day. Since these fractions are probably also composed of rapidly and slowly metabolized proteins, the microsomal fractions probably contain proteins which are replaced in a matter of a few hours.

In other organs, particularly the liver, the microsomal fractions have shown the highest rate of amino acid incorporation in *in vivo* and *in vitro* experiments (BORSOOK *et al.* 1950; HULTIN, 1950). This fraction is therefore assumed to play a major role in protein synthesis (KELLER, ZAMECNIK, and LOFTFIELD, 1954). The microsomal fractions of the various parts of the central nervous system behave in a manner similar to those of the liver. The conclusion is of particular interest for the nervous system since Nissl granules are assumed on the basis of electron microscopic studies (PALAY

and PALADE, 1955) to correspond to the microsomes of the neurons. Changes in the basophilic granules of the neuronal cells under pathological and stress situations have been described as well as the disintegration of the microsomes of the brain of cats perfused with glucose-free perfusion fluids (ABOOD and GEIGER, 1955). In view of the fast turnover of the microsomal proteins it is not surprising that this particulate fraction will respond rapidly to changes in metabolism produced by bodily and/or environmental influences.

While the 'microsomal' proteins belong to the protein fractions with the highest rate of amino incorporation, the protein moiety of the proteolipids apparently has a very slow metabolism (Table 10). The rate of incorporation of [^{14}C]-lysine into

TABLE 10.—COMPARISON OF SPECIFIC ACTIVITY OF THE PROTEOLIPIDS WITH THE REST OF THE BRAIN PROTEIN

	Experiment I					Experiment II		
	1	2	3	4	5	6	7	8
	Specific activity (counts/μg lysine/min)			Ratio	Ratio	Specific activity (counts/μg lysine/min)		Ratio
	RP*	PL† A + B	PL C	1/2	1/3	RP	PL A + B + C	6/7
Cortex	1·7	0·2	0·13	7·4	13·2	1·90	0·21	9·0
Cerebellum	1·2	0·1	0·03	12·4	42·8	1·67	0·22	7·6
White	0·8	0·03	0·02	25·1	34·0	1·10	0·06	18·9
Hindbrain and midbrain	1·1	0·07	0·11	16·9	10·2	1·21	0·15	8·2
Rest of brain	1·5	0·19	0·10	7·8	14·2	1·53	0·15	10·0

* RP = rest of proteins.
† PL = proteolipids.

proteolipids A and B and C respectively is ten to forty times slower than into the rest of the brain proteins. Since the proteolipids are essentially components of the myelin structures of the central nervous system this finding underscores the great metabolic stability of myelin, a conclusion already suggested by the studies of the rejuvenation of the fatty acids in the brain of adult rats (WAELSCH, SPERRY and STOYANOFF, 1941).

The findings reported in this paper show that the heterogeneity of turnover rates of the proteins of the central nervous system is attributable primarily to the different rates of metabolism of the cellular fractions. The spectrum of turnover rates is extended by proteins with varying turnover rates in different areas of the brain, and by specific proteins such as those occurring in the proteolipids.

SUMMARY

The specific activity of the free lysine and of the protein bound lysine of different areas and of different cellular fractions from these areas was determined as a function of time in short term experiments in monkey brains after the administration of [^{14}C]-lysine. The specific activity of the free lysine of the corpus callosum and its radiation was very low, while the specific activity of the free lysine of predominantly

grey structures was considerably higher. The turnover rates of the protein in the cortex, cerebellum, thalamus, and hypothalamus were higher than the turnover rate of the proteins of the spinal cord. The highest turnover rate was shown by the proteins of the corpus callosum and its radiation, a result which is interpreted as due to the presence of different compartments of free lysine in the white matter.

Of the cellular elements, the proteins of the fraction sedimented at a centrifugal force characteristic for microsomes showed the highest incorporation of lysine. The results are discussed in terms of their significance for protein synthesis, and for the role of the Nissl granules. Data on the content of free lysine of the different areas, and of the protein-bound lysine of different areas and cellular fractions are reported.

Acknowledgement—This work was supported by grants from the National Institute of Neurological Diseases and Blindness (Grants B-226 and B-557) of the National Institutes of Health, Public Health Service, Scottish Rites Supreme Council, and by a contract between the Office of Naval Research and the Psychiatric Institute. Preliminary reports have appeared (LAJTHA and FURST, 1957; WAELSCH, 1957).

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ÜBER DAS VORKOMMEN DER $\Delta^{9,12,15,18}$-*n*-TETRAKOSA-TETRAENSÄURE IN DEN GLYCERINPHOSPHATIDEN DES GEHIRNS UND DEREN ISOLIERUNG

THE ISOLATION OF $\Delta^{9,12,15,18}$-*n*-TETRACOSANETETRAENOIC ACID FROM THE PHOSPHATIDES OF THE BRAIN

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(*Received* 31 *August* 1957)

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NACH der Auffindung der C_{22}-Polyensäuren in den Gehirnphosphatiden durch den einen von uns (KLENK, 1930) wurde kurz darauf auch festgestellt (KLENK, 1932), dass die Gehirnphosphatide, wenn überhaupt, so jedenfalls nur sehr geringe Mengen C_{18}-Polyensäuren enthalten und dass im Wesentlichen nur C_{20}- und C_{22}-Polyensäuren vorliegen, während man bis dahin angenommen hatte, dass es sich um C_{18}- und C_{20}-Polyensäuren handelt. Inzwischen konnten die meisten dieser C_{20}- und C_{22}-Polyensäuren isoliert und in ihrer Struktur aufgeklärt werden (KLENK und BONGARD, 1952*a*; KLENK und LINDLAR 1955*b*). Es ergab sich, dass ihnen durchweg Divinylmethanstruktur zukommt und dass sie so gut wie ausschliesslich dem Linol- und Linolensäuretyp angehören.

Im Laufe dieser Untersuchungen wurden auch Anzeichen für das Vorkommen von kleinen Mengen C_{24}-Polyensäuren erhalten. Sie fanden sich bei der fraktionierten Vakuumdestillation der Methylester in der höchst-siedenden Fraktion. Trotzdem sich bei dieser Fraktion stärkere Zersetzungserscheinungen zeigten, gelang es doch daraus durch Gegenstromverteilung noch eine einigermassen einheitliche C_{24}-Tetraensäure zu gewinnen.

Die chromatographische Analyse der bei dem oxydativen Ozonidabbau der freien Säure und des Esters auftretenden Abbaudicarbonsäuren nach dem Verfahren von KLENK und BONGARD (1952*b*) führte zu dem in der Tabelle 1 zusammengestellten Ergebnis.

Bei der reduktiven Ozonidspaltung der C_{24}-Tetraensäure nach einem vor kurzem erst ausgearbeitetem Verfahren, (KLENK und BROCKERHOFF, 1958), wobei die auftretenden Abbaualdehyde in die 2,4-Dinitrophenylhydrazone übergeführt, diese chromatographisch getrennt und quantitativ bestimmt wurden, erhielt man neben dem Hydrazon des Acetaldehyds, der durch Zerfall des als eigentliches Abbauprodukt auftretenden Malondialdehyds (oder Formylessigsäure) entstanden sein dürfte, noch 0·07 Mol Propionaldehyd und 0·80 Mol Capronaldehyd pro Mol Polyensäure.

Aus diesem Abbauergebnis geht hervor, dass es sich im Wesentlichen um $\Delta^{9,12,15,18}$-Tetrakosatetraensäure handelt. Sie bildet offensichtlich den Hauptteil der C_{24}-Polyensäuren und ist die dritte der in den Glycerinphosphatiden aufgefundenen Tetraensäuren vom Linolsäuretyp.

TABELLE 1.—ABBAUDICARBONSÄUREN DER C_{24}-TETRAENSÄURE
Menge in Mol pro Mol Polyensäure

	Azelainsäure (C_7)		C_5-C_7-Dicarbons.		Malonsäure (C_3)
	freie Säure	Halb-Ester	freie Säure	Halb-Ester	freie Säure
C_{24}-Tetraens.	0·74	—	0·1	—	1·46
Methylester	—	0·84	0·05	—	1·66

TABELLE 2.—DIE TETRAENSÄUREN DER GLYCERINPHOSPHATIDE DES GEHIRNS

	Lage der Doppelbindungen von der endständigen CH_3-Gruppe aus gerechnet
$\Delta^{5,8,11,14}$-Eikosatetraens. (KLENK und LINDLAR, 1955*b*)	6 9 12 15
$\Delta^{7,10,13,16}$-Dokosatetraens. (KLENK und LINDLAR, 1955*a*)	6 9 12 15
$\Delta^{9,12,15,18}$-Tetrakosatetraens.	6 9 12 15

Die Zusammenstellung in Tabelle 2 zeigt dass alle drei Polyensäuren sich gegenseitig sehr nahe stehen. Die eine könnte in die andere durch Abspaltung oder Anlagerung von Essigsäure auf der Seite der Carboxylgruppe übergehen, ohne dass sich dabei die Lage der Doppelbindungen verändert.

BESCHREIBUNG DER VERSUCHE

Aus 881 g ätherlöslichen Glycerinphosphatiden von Menschengehirn wurden wie früher beschrieben (KLENK und BONGARD, 1952*a*) 139g hochungesättigte Fettsäuren (Jdz. 300) gewonnen und die Methylester anschliessend unter Benützung einer 750 mm hohen, mit kleinen Drahtnetzsätteln gefüllten Kolonne (ϕ 29 mm) mit verspiegelten Vakummantel in zwei Einzelportionen von je 70 g im Hochvakuum (10^{-4} mm Hg, am Kopf der Kolonne gemessen) fraktioniert destilliert (KLENK, 1955). Nachdem die C_{16}, C_{18}, C_{20} und auch ein Teil der C_{22}-Fettsäureester abdeststilliert war, verblieb von beiden Ansätzen ein Rückstand von insgesamt 66 g, der jetzt in derselben Weise mit Hilfe einer kleineren, nur 350 mm hohen Kolonne mit dem in Tab. 3 zusammengestellten Ergebnis weiter fraktioniert destilliert wurde, wobei man schliesslich Fraktion 9 ohne Anwendung einer Kolonne direkt aus dem Destillationskolben herausdestillierte. Menge des Destillationsrückstands 9 g.

Die Fraktionen 1–7 (farblose, leicht flüssige Öle) erwiesen sich als praktisch reine C_{22}-Polyensäuren, deren Hydrierungsprodukte mit *n*-Dokosansäure (Schmp. 79·0–79·6°) identisch waren, auch die papierchromatographische Prüfung nach KAUFMANN und NITSCH (1954) in der Modifikation von WAGNER, ABISCH und BERNHARD (1955) ergab Identität mit dieser Säure. Bei Fraktion 8 (hydrierte Säure) zeigte sich papierchromatographisch eine Beimengung von etwa 20% Tetrakosansäure ($R_{\text{Dokos.}} = 0{\cdot}60$*) während bei Fraktion 9 (hydrierte Säure) es sich im Wesentlichen nur noch um *n*-Tetrakosansäure handelte.

Aus Fraktion 9, welche als gelbe, zähe Flüssigkeit überging und nach einiger Zeit

TABELLE 3.—FRAKTIONIERTE DESTILLATION DER HÖHER SIEDENDEN ANTEILE DES POLYENSÄUREESTERGEMISCHS DER GEHIRNPHOSPHATIDE

	Badtemp. (°C)	Methylester			hydrierte Säuren	
		Sdp. (10^{-4} mm)	Menge (g)	Jdz.*	Schmp.†	Aquiv. gew.†
(1)	215	115	6·1	320	78·8 (79·2)	334 (340)
(2)	218	119	5·4	321	78·8 (79·2)	334 (340)
(3)	220	125	5·0	328	79·4 (79·4)	340 (341)
(4)	223	126	5·9	326	78·8 (79·6)	340 (342)
(5)	228	127	7·0	325	79·2 (80·5)	340 (342)
(6)	230	124	6·8	327	79·0 (80·0)	340 (341)
(7)	234	132	6·9	325	77·6 (78·9)	341 (340)
(8)	250	130	7·5	302	74·0 (75·0)	344 (346)
(9)	270	150	6·0	218	78·4 (81·6)	360 (371)

* Nach ROSENMUND und KUHNHENN
† Die eingeklammerten Zahlen gelten für die umkristallisierten Säuren.

mit Kristallen (Cholesterin) durchsetzt war, wurden durch Verseifung und Abtrennung des Unverseifbaren 4·1g freie Säuren erhaltren. Wie aus den UV-Spektren (Abb. 1) ersichtlich enthält die Substanz schon vor der Alkaliisomerisation beträchtliche Mengen konjugiert ungesättigte Fettsäuren, die wohl sekundär bei der Destillation entstanden sein dürften.

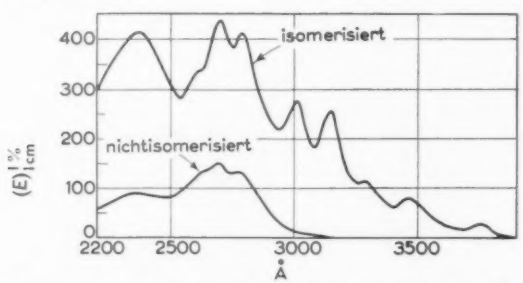

ABB 1.—UV-Spektren des C_{24}-Polyensäuregemischs vor und nach der Alkaliisomerisation (21% KOH–Glykol, 8 min, 180°).

Die Gegenstromverteilung führte man wie früher nach dem Verfahren von AHRENS und CRAIG (1952) in einer 200-stufigen vollautomatischen Apparatur aus. Abb. 2 zeigt die Verteilungskurve. Während die Fraktion 200–470 in der Hauptsache C_{22}-Polyensäuren (nach der Hydrierung papierchromatographisch als *n*-Dokosansäure identifiziert) enthielten, bestand die Fr. 490–520 so gut wie ausschließlich aus C_{24}-Säuren (nach der Hydrierung papierchromatographisch als *n*-Tetrakosansäure identifiziert).

Die C_{24}-Tetraensäure wurde aus den vereinigten Fraktionen 496–510 gewonnen. 0·35 g gelbes, leicht flüssiges Öl. Jdz. 266; $C_{24}H_{40}O_2$ ber. 276. Die Säure ist papierchromatographisch einheitlich. Die Wanderungsgeschwindigkeit ist geringer als die der beiden anderen Tetraensäuren. Verglichen mit Dokosatetraensäure ($R = 1·00$)

* Relative Wanderungsgeschwindigkeit gegenüber *n*-Dokosans.

war für die Tetrakosatetraensäure $R_D = 0{\cdot}63$ und für die Eikosatetraensäure $R_D = 1{\cdot}44$. Dem grossen Unterschied dieser Werte entsprechend ist ein Gemisch der drei Säuren papierchromatographisch scharf in die Komponenten zu trennen.

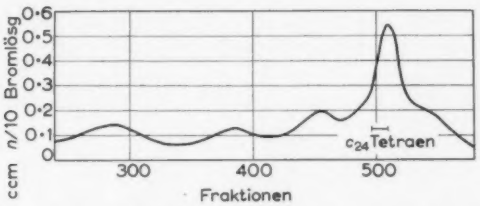

ABB. 2.—Verteilungskurve der C_{24}-Polyensäurefraktion.

Abb. 3 zeigt die UV-Spektren vor und nach der Alkaliisomerisation. Das Hydrierungsprodukt schmolz bei 83·0–83·4° und gab mit *n*-Tetrakosansäure (Schmp. 83·2–83·8°) keine Schmelzpunktdepression. Eine Mischprobe mit *n*-Dokosansäure (79·0–79·6°) zeigte dagegen eine deutliche Depression. Äquiv. gew. 370/372; $C_{24}H_{48}O_2$ ber. 368·6

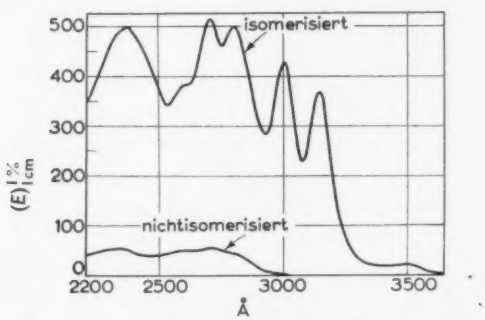

ABB. 3.—UV-Absorbtionskurven der Tetrakosatetraensäure vor und nach der Alkaliisomerisation

Die oxydative Ozonidspaltung

Die Zusammensetzung der (a) aus 9·07 mg Tetraensäure und (b) aus 11·35 mg Methylester wie üblich (KLENK und BONGARD, 1952*b*; KLENK und MONTAG, 1957) erhaltenen Abbaudicarbonsäuren zeigen die Abb. 4 und 5. Bei (a) entfernte man vor der chromatographischen Analyse aus den Abbausäuren die Monocarbonsäuren durch Behandlung mit Petroläther, während bei (b) infolge des Vorhandenseins der Dicarbonsäurehalbester die Petrolätherbehandlung unterblieb und die Abbausäuren als solche für die säulenchromatische Analyse eingesetzt wurden. Die papierchromatographische Prüfung ergab bei (a) für Fraktion 5–22 reine Azelainsäure, für Fraktion 24–35 Azelainsäure mit kleineren Beimengungen niedrigerer Dicarbonsäuren und zwar Pimelin- und Adipinsäure (nach KLENK und FAILLARD, 1953), für Fraktion 42–53 Pimelin-, Adipin- und Glutarsäure und für Fraktion 67–91 Malonsäure (nach LUGG und OVERELL, 1947). Fraktion 62–65 konnte nicht identifiziert werden. Die aus Fraktion 5–22 isolierte Azelainsäure schmolz nach Umkristallisieren aus Wasser bei 98° und gab mit Testazelainsäure (Schmp. 104°) keine Schmelzpunktdepression. Äquiv. gew. 99; $C_9H_{16}O_4$ ber. 94.

Bei (b) ergab die papierchromatographische Prüfung Fraktion 3–18 nach Verseifung der Halbester wieder Azelainsäure mit Spuren Beimengungen von niedrigeren Dicarbonsäuren (nach KLENK und FAILLARD, 1953) und für Fraktion 74–97 Malonsäure (nach LUGG und OVERELL, 1947). Die Fraktionen 47–59 und 63–73 konnten nicht identifiziert werden.

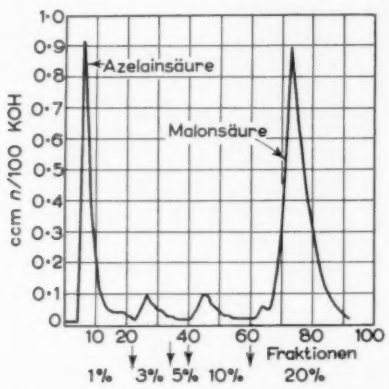

ABB. 4.—Chromatographische Trennung der Abbaudicarbon-säuren der Tetrakosatetraensäure durch Säule D.
1% (5–22) : 3·32 ccm n/100-KOH, gef. 0·66 Mol Azelainsäure.—3% (24–35) : 0·41 ccm n/100-KOH, gef. 0·08 Mol Azelainsäure.—10% (42–53) : 0·51 ccm n/100-KOH, gef. 0·1 Mol C_5, C_6 u. C_7 Dicarbonsäuren.—20% (62–65) : 0·12 ccm n/100-KOH, gef. 0·02 Mol Dicarbonsäure (nicht identifiziert).—20% (67–91) : 7·33 ccm n/100-KOH, gef. 1·46 Mol Malonsäure

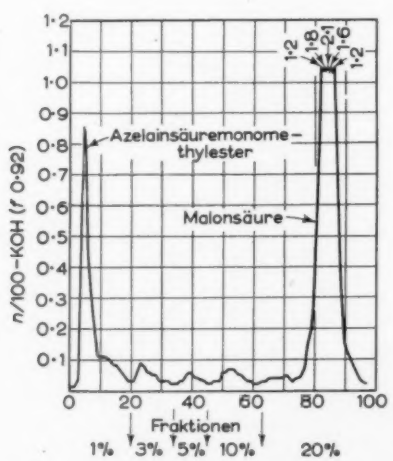

ABB. 5.—Chromatographische Trennung der Abbausäuren des Tetrakosatetraensäuremethylesters durch Säule D.
1% (3–18): 2·9 ccm n/100-KOH + 2·4 ccm n/100-KOH durch Verseifen, gef. 0·79 Mol Azelainsäuremonomethylester.—3% (21–33): 0·3 ccm n/100-KOH, gef. 0·05 Mol Azelainsäure.—10% (47–59) : 0·33 ccm n/100-KOH, gef. 0·05 Mol Dicarbonsäure (nicht identifiziert).—20% (63–73) : 0·23 ccm n/100-KOH, gef. 0·04 Mol Dicarbonsäure (nicht identifiziert).—20% (74–97) : 10·3 ccm n/100-KOH, gef. 1·66 Mol Malonsäure

Die reduktive Ozonidspaltung

Der Abbau von 10·7 mg Tetraensäure nach dem Verfahren von KLENK und BROCKERHOFF (1958) führte zu den in Tabelle 4 zusammengestellten Ergebnis. Die

TABELLE 4.—ABBAUALDEHYDE DER TETRAKOSATETRAENSÄURE

		Extinktion	Menge Hydrazone	
			mg	Mol pro Mol Polyensäure
Zone 1	Hydrazone von Acetaldehyd	nicht bestimmt		
Zone 2	Propionaldehyd	0·02	0·5	0·071
Zone 3	Capronaldehyd	0·266	6·7	0·80

säulenchromatographisch getrennten Hydrazone waren papierchromatographisch einheitlich und mit den Testsubstanzen identisch.

Die Untersuchung wurde durch Mittel der deutschen Forschungsgemeinschaft unterstützt.

ZUSAMMENFASSUNG

Aus dem Polyensäuregemisch der Glycerinphosphatide des Gehirns wurde die $\Delta^{9,12,15,18}$-Tetrakosatetraensäure isoliert. Die Polyensäure findet sich im Gemisch nur in kleinen Mengen (ca. 1–2%). Sie ist eine höhere Homologe der $\Delta^{7,10,13,16}$-Dokosatetraensäure und der $\Delta^{5,8,11,14}$-Eikosatetraensäure (Arachidonsäure), welche beide Hauptbestandteile des Gemischs sind. Die Struktur wurde durch oxydativen und reduktiven Ozonidabbau aufgeklärt, wobei als hauptsächlichste Abbauprodukte Azelainsäure, Malonsäure, und Capronaldehyd auftraten.

SUMMARY

$\Delta^{9,12,15,18}$-Tetracosanetetraenoic acid has been isolated from the mixture of polyenoic acids obtained from the glycerophosphatides of ox brain: it is present only in small quantities (about 1–2 per cent of the total mixed fatty acids). It is a higher homologue of $\Delta^{7,10,13,16}$-docosanetetraenoic acid and $\Delta^{5,8,11,14}$-eicosanetetraenoic (arachidonic) acids, which are the main constituents of the mixture. The structure was established by oxidative degradation of the ozonides, from which the main products were azelaic and malonic acids and caproic aldehyde.

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ÜBER DIE C_{22}-POLYENSÄUREN DER GLYCERINPHOSPHATIDE DES GEHIRNS

THE C_{22}-POLYENOIC ACIDS OF THE PHOSPHATIDES OF THE BRAIN

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(*Received* 23 *September* 1957)

OL.
2
7/58

Aus dem C_{22}-Polyensäuregemisch der Glycerinphosphatide des Gehirns wurden von KLENK u. LINDLAR (1951*a*) bereits $\Delta^{7,10,13,16}$-Dokosatetraensäure und $\Delta^{4,7,10,13,16,19}$-Dokosahexaensäure, die beiden Hauptkomponenten des Gemischs, herausfraktioniert. Nachgewiesen wurde auch das Vorhandensein der $\Delta^{4,7,10,13,16}$-Dokosapentaensäure und eine Dokosatriensäure in kleineren Mengen. Es handelte sich damals um den ersten Versuch, ein derartiges Gemisch durch Gegenstromverteilung nach dem Verfahren von AHRENS und CRAIG (1952) in die Komponenten zu zerlegen. Da nur eine kleinere, behelfsmässige Apparatur mit 36 Elementen zur Verfügung stand, liess die Trennwirkung noch etwas zu wünschen übrig, so dass die in geringeren Mengen vorhandenen Komponenten des Gemischs nicht in den für die genauere Untersuchung ausreichenden Mengen anfielen.

Wir haben nun diese Versuche wiederholt und zur Trennung eine vollautomatische 200-stufige Apparatur benützt. Auf diese Weise gelang es, eine praktisch reine, völlig hexaensäurefreie Pentaensäure und eine allerdings, noch etwas unreine Triensäurefraktion zu erhalten. Die einzelnen Komponenten des Gemischs wunden jetzt soweit getrennt, dass eine Berechnung der annähernden Zusammensetzung des C_{22}-Polyensäuregemischs möglich ist. Das Gemisch enthält demnach: Hexaensäure 43%, Pentaensäure 6%, Tetraensäure 38%, Triensäure 8%, Diensäure u. nicht identifizierter Anteil 5%.

Zur Bestimmung der Lage der Doppelbindungen in der Trien- und Pentaensäure wurde sowohl der oxydative wie auch der reduktive Ozonidabbau durchgeführt und die Zusammensetzung der Abbauprodukte durch chromatographische Analyse bestimmt. Das Ergebnis ist in den Tab. 1 und 2 zusammengestellt.

Als hauptsächlichste Abbauprodukte wurden wie die Tabellen zeigen bei der Triensäure Pimelinsäure, Malonsäure und Pelargonaldehyd aufgefunden. Es liegt demnach $\Delta^{7,10,13}$-Dokosatriensäure vor. Die Säure ist noch mit etwas C_{24}-Polyensäure verunreinigt, wie die chromatographische Prüfung des Hydrierungsprodukts ergab. Da das UV-Spektrum der isomerisierten Substanz die Anwesenheit einer kleinen Menge Tetraensäure (ca. 10%) anzeigt, ist anzunehmen, dass es sich um die vor kurzem (KLENK u. MONTAG, 1958) aufgefundene $\Delta^{9,12,15,18}$-Tetrakosatetraensäure handelt, wodurch sich auch das Auftreten der kleinen Menge Azelainsäure und des Capronaldehyds unter den Abbauprodukten erklärt. Die ebenfalls in kleinen Mengen

TABELLE 1.—OXYDATIVER OZONIDABBAU DER C_{22}-TRIENSÄURE UND DER C_{22}-PENTAENSÄURE. MENGE DER ABBAUDICARBONSÄUREN IN MOL PRO MOL POLYENSÄURE

	Sebacinsäure (C_{10})		Pimelinsäure (C_7)		Bernsteinsäure		Malonsäure
	freie Säure	Halbester	freie Säure	Halbester	freie Säure	Halbester	freie Saure
Triensäure	0·12*	—	0·79	—	—	—	1·04
Methylester	—	siehe†	0·08	0·80†	—	—	0·98
Pentaensäure	—	—	0·26	—	0·60	—	1·98
Methylester	—	—	0·05	siehe‡	0·11	0·82‡	2·10

* Enthält noch etwas Azelainsäure (C_9) und Pimelinsäure.
† Enthält noch etwas Halbester der höheren Dicarbonsäuren.
‡ Enthält noch Halbester der Pimelinsäure.

vorgefundene Sebacinsäure ist möglicherweise auf eine weitere Verunreinigung mit $\Delta^{10,13,16}$-Dokosatriensäure oder $\Delta^{10,13}$-Dokosadiensäure zurückzuführen.

Die Pentaensäure lieferte als hauptsächlichste Abbauprodukte Bernsteinsäure, Malonsäure und Capronaldehyd, so dass es sich hier im wesentlichen um $\Delta^{4,7,10,13,16}$-Dokosapentaensäure handelt, deren Vorhandensein schon früher nachgewiesen wurde (KLENK u. BONGARD (1952*b*); KLENK u. LINDLAR (1955*a*)). Da als weitere Abbauprodukte Pimelinsäure und Propionaldehyd und zwar beide in einer Menge von etwa 0·2 Mol pro Mol Polyensäure aufgefunden wurden, kann kaum ein Zweifel darüber bestehen, dass das isolierte Pentaensäurepräparat noch die isomere $\Delta^{7,10,13,16,19}$-Dokosapentaensäure in einer Menge von 20–30% enthält. Die Letztere ist in den Phosphatiden der Rinderleber der Hauptbestandteil des C_{22}-Polyensäuregemischs (KLENK u. TOMUSCHAT, 1957). Die Möglichkeit, dass der Propionaldehyd von einer Beimengung von Hexaensäure stammt, ist auf Grund des spektrophotometrischen Befunds auszuschliessen.

TABELLE 2.—REDUKTIVER OZONIDABBAU DER C_{22}-TRIENSÄURE UND DER C_{22}-PENTAENSÄURE. MENGE DER 2:4-DINITROPHENYLHYDRAZONE DER ABBAUALDEHYDE IN MOL PRO MOL POLYENSÄURE

	2:4-Dinitrophenylhydrazone von		
	Propionaldehyd (C_3)	Capronaldehyd (C_6)	Pelargonaldehyd (C_9)
Triensäure	—	0·15	Hauptmenge*
Pentaensäure	0·2	0·6	—

* Nicht quantitativ bestimmt.

Die vorliegende Arbeit bringt die Untersuchungen (KLENK u. BONGARD (1952*b*); KLENK u. LINDLAR (1955*a*, *b*) KLENK u. MONTAG (1958)) über die Polyensäuren der Glycerinphosphatide des Gehirns zu einem gewissen Abschluss. In der Tab. 3 sind alle bisher aufgefundenen Polyensäuren zusammengestellt. Das Vorkommen

TABELLE 3.—ZUSAMMENSTELLUNG DER BISHER IN DEN GLYCERINPHOSPHATIDEN DES GEHIRNS AUFGEFUNDENEN POLYENSÄUREN

	Ölsäuretyp	Linolsäuretyp	Linolensäuretyp
C_{20}	$\Delta^{5,8,11}$-Triens.	$\Delta^{11,14}$-Diens. $\Delta^{8,11,14}$-Triens. $\Delta^{5,8,11,14}$-Tetraens.	
C_{22}	$\Delta^{7,10,13}$-Triens.	$\Delta^{7,10,13,16}$-Tetraens. $\Delta^{4,7,10,13,16}$-Pentaens.	$\Delta^{7,10,13,16,19}$-Pentaens. $\Delta^{4,7,10,13,16,19}$-Hexaens.
C_{24}		$\Delta^{9,12,15,18}$-Tetraens.	

einiger anderer in der Tabelle nicht aufgeführten Polyensäuren in kleinen Mengen kann als wahrscheinlich angesehen werden.

BESCHREIBUNG DER VERSUCHE

Als Ausgangsprodukt für die vorliegenden Untersuchungen dienten die Fr. 4–6 der Tab. 3 von der früheren Arbeit (KLENK u. MONTAG, 1958). Sie wurden durch fraktionierte Vakuumdestillation aus den höher siedenden Anteilen der Polyensäuremethylester der Glycerinphosphatide des Gehirns gewonnen und enthielten praktisch nur C_{22}-Polyensäuren. Abb. 1 zeigt die UV-Absorptionsspektren vor und nach der Alkaliisomerisation. Aus der letzteren ergibt sich, dass der Hauptteil des Gemischs aus Tetraen- und Hexaensäuren bestand.

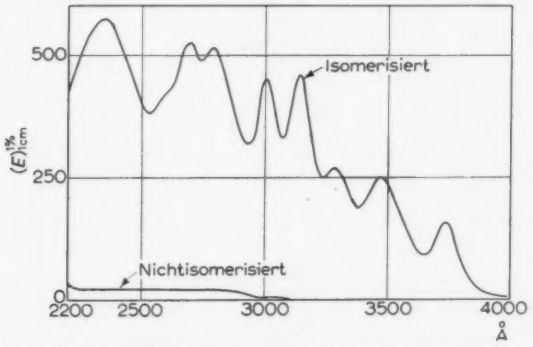

ABB. 1.—UV-Absorptionsspektren des C_{22}-Polyensäuregemischs vor und nach der Alkaliisomerisation (21% KOH-Glykol, 8 Min., 180°).

18 g freie Säuren der vereinigten Fr. 4–6 wurden nun in der üblichen Weise (KLENK u. MONTAG, 1957*a*) durch Gegenstromverteilung unter Verwendung eines vollautomatischen 200-stufigen Geräts in die Komponenten zerlegt (Abb. 2).

Zur Abgrenzung der verschiedenen Polyensäuren gegeneinander wurden einzelnen

Fraktionen Proben entnommen von der daraus nach Abdampfen des Lösungsmittels erhaltenen Substanz nach Alkaliisomerisierung das UV-Absorptionsspektrum aufgenommen. Es ergab sich folgende Verteilung: Fr. 240–428 Hexaensäure, Fr.

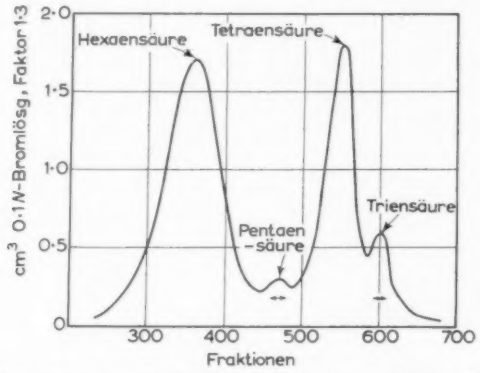

ABB. 2.—Verteilungskurve des C_{22}-Polyensäuregemischs. Ordinate: Bromverbrach von 2 cm³ Proben jeder 2. Fraktion.

429–488 Pentaensäure, Fr. 489–584 Tetraensäure, Fr. 585–614 Triensäure und Fr. 615–680 Diensäure (stark verunreinigt). Tab. 4 enthält die auf Grund des Bromverbrauchs dieser Fraktionen berechnete Zusammensatzung des C_{22}-Polyensäuregemischs.

TABELLE 4.—ZUSAMMENSETZUNG DES C_{22}-POLYENSÄUREGEMISCHS

	Jodz. der isolierten Säuren	Verbrauch 0·1 n. Bromlösg. (cc)	Menge der Fettsäuren	
			(g)	(%)
Hexaensäure	444	2,380	6·8	43
Pentaensäure	333	258	0·98	6
Tetraensäure	300	1,416	6·0	38
Triensäure	232	223	1·24	8
Diensäure	161	92	0·73*	5

* Zur Berechnung wurde angenommen, dass hier eine Diensäure vorliegt.

Dokosatriensäure

Zur Untersuchung kamen die vereinigten Fr. 593–608; 0·65 g, schwach gelbes, dünnflüssiges Öl. Jodz. 232 (nach ROSENMUND u. KUHNHENN, 1923), $C_{22}H_{38}O_2$ ber. 228. Die Abb. 3 zeigt die UV-Absorptionsspektren vor und nach Alkaliisomerisierung. Demnach enthält die Substanz noch etwa 10% Tetraensäure. Das Hydrierungsprodukt schmolz bei 78–78·4° und gab mit *n*-Dokosansäure (Schmp. 79·5–80°) keine Schmelzpunktsdepression. Die papierchromatographische Prüfung (Methode von KAUFMANN u. NITSCH, 1954, modifiziert von WAGNER, ABISCH u. BERNHARD, 1955) ergab *n*-Dokosansäure mit einer kleinen Beimengung von *n*-Tetrakosansäure.

Die oxydative Ozonidspaltung. Die Zusammensetzung der (a) aus 12·3 mg Triensäure und (b) aus 12·69 mg Methylester der wie üblich (KLENK u. BONGARD, 1952*a*) erhaltenen Abbaudicarbonsäuren zeigen die Abb. 4 und 5. Bei (a) waren die

Monocarbonsäuren durch Behandlung mit Petroläther entfernt, nicht dagegen bei (b). Die papierchromatographische Prüfung ergab bei (a) für Fr. 4–10 hauptsächlich Sebacinsäure, ausserdem Pimilin- und Azelainsäure, für Fr. 16–30 Pimelinsäure,

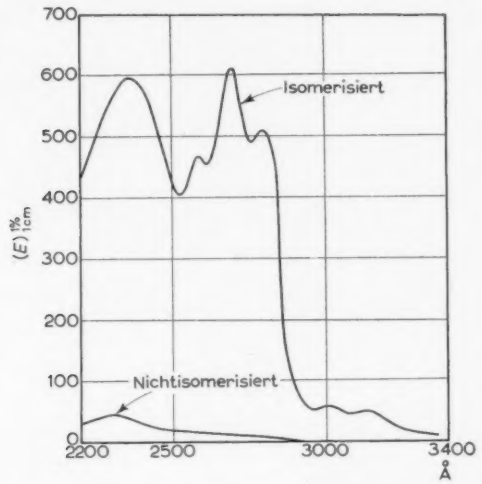

ABB. 3.—UV-Absorptionsspektren der Dokosatriensäure vor und nach der Alkaliisomerisation.

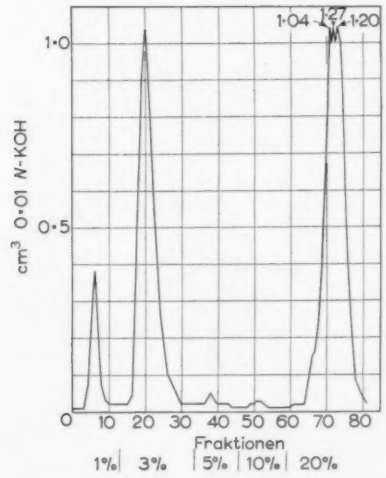

ABB. 4.—Chromatogramm der Abbaudicarbonsäuren der Dokosatriensäure mit Säule D. 1% (Fr. 4–10): 0·88 cc 0·01N-KOH, gef. 0·12 Mol Sebacinsäure. 3% (Fr. 16–30): 5·14 cm³ 0·01 N-KOH, gef. 0·7 Mol Pimelinsäure. 5% (Fr. 32–43): 0·09 cm³ 0·01 N-KOH, gef. 0·09 Mol Pimelinsäure. 20% (Fr. 60–80): 7·65 cm³ 0·01 N-KOH gef. 1·04 Mol Malonsäure.

spurenweise Azelain- und Sebazinsäure, für Fr. 32–43 Pimelinsäure und für Fr. 60–80 Malonsäure. Bei (b) war das Ergebnis entsprechend.

Die reduktive Ozonidspaltung. Der Abbau von 6·6 mg Triensäure nach dem Verfahren von KLENK u. BROCKERHOFF (1957), wobei von der gegebenen Vorschrift (zur besseren Erfassung des Pelargonaldehyds) insofern abgewichen wurde als das Abdestillieren der Abbaualdehyde unterblieb, führte zu dem 2:4-Dinitrophenyl-

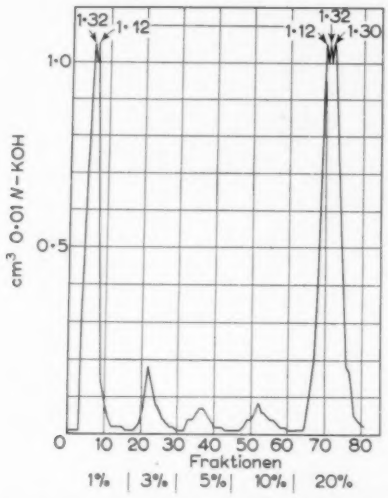

ABB. 5.—Chromatogramm der Abbausäuren des Dokosatriensäuremethylesters mit Säule D. 1% (Fr. 4–12); 4·38 cm³ 0·01 N-KOH + 2·92 cm³ 0·01 N-KOH durch Verseifen, gef. 0·8 Mol Pimelinsäure-monomethyl-ester. 3% (19–30): 0·58 cm³ 0·01 N-KOH, gef. 0·08 Mol Pimelinsäure. 5% (Fr. 33–40): 0·29 cc 0·01 N-KOH, gef. 0·04 Mol Dicarbonsäure (nicht identifiziert). 10% (Fr. 48–60): 0·36 cc 0·01 N-KOH, gef. 0·05 Mol Dicarbonsäure (nicht identifiziert). 20% (Fr. 66–80): 7·19 cc 0·01 N-KOH, gef 0·98 Mol Malonsäure.

hydrazon des Pelargonaldehyds als Hauptprodukt, dessen quantitative Bestimmung jedoch nicht ausgeführt wurde und 0·83 mg (0·15 Mol) Hydrazon des Capronaldehyds. Unter dem Hydrazongemisch der Abbaualdehyde fand sich auch Acetaldehyd-hydrazon, das jedoch nicht quantitativ bestimmt wurde. Die säulenchromatographisch getrennten Hydrazone waren papierchromatographisch einheitlich und mit den Testsubstanzen identisch.

Dokosapentaensäure

Zur Untersuchung kamen die vereinigten Fr. 462–478. Nahezu farbloses, dünnflüssiges Öl (0·30 g). Jodz. 333, $C_{22}H_{34}O_2$ ber. 385*. Die Abb. 6 zeigt die UV-Spektren vor und nach der Alkaliisomerisierung. Die spez. Extinktion im Pentaen- und Tetraenbereich liegen merklich niedriger als die der $\Delta^{7,10,13,16,19}$-Dokosapentaensäure (für λ 346 mμ $[E]^{1\%}_{1cm} = 420$ gegenüber 605), welche aus Phosphatiden der Rinderleber isoliert werden konnte (KLENK u. TOMUSCHAT, 1957). Ob diese Abweichung auf die Unterschiede in der Struktur zurückzuführen ist, bedarf noch der weiteren Aufklärung. Das Hydrierungsprodukt schmolz bei 81–81·6° und gab mit *n*-Dokosansäure (Schmp. 79·5–80°) keine Schmelzpunktsdepression. Papierchromatographisch verhielt sich die Substanz wie reine *n*-Dokosansäure.

Die oxydative Ozonidspaltung. Die Zusammensetzung der (a) aus 10·72 mg

* Die Methode von ROSENMUND u. KUHNHENN gibt bei den hochungesättigten Fettsäuren zu niedrige Werte.

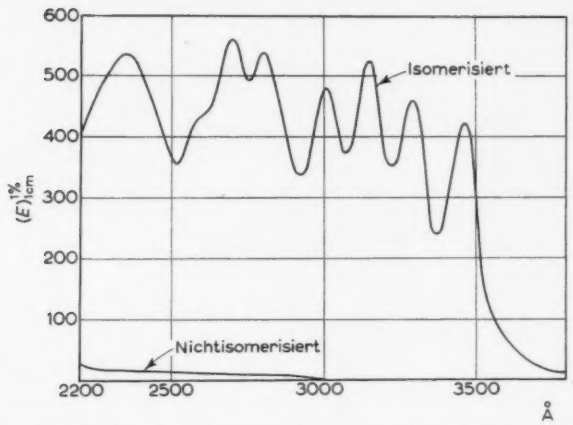

ABB. 6.—UV-Absorptionsspektren der Dokosapentaensäure vor und nach der Alkaliisomerisation.

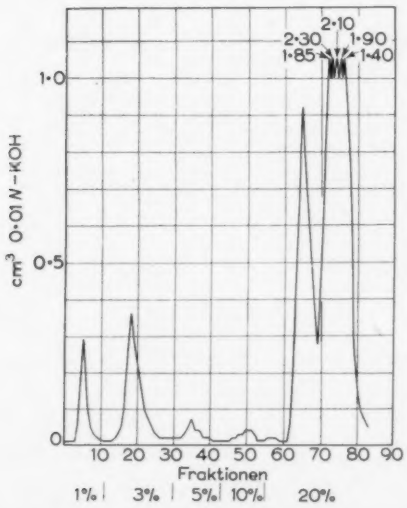

ABB. 7.—Chromatogramm der Abbaudicarbonsäuren der Dokosapentaensäure mit Säule D. 1% (Fr. 4–10): 0·52 cc 0·01 N-KOH, gef. 0·08 Mol nicht identifizierter Vorlauf. 3 + 5% (Fr. 15–40): 1·69 cm³ 0·01 N-KOH, gef. 0·26 Mol Pimelinsäure. 10% (Fr. 45–53): 0·13 cm³ 0·01 N-KOH gef. 0·02 Mol nich identifierte Dicarbonsäuren. 20% (Fr. 62–68): 3·9 cm³ 0·01 N-KOH,gef.0·6 Mol Bernsteinsäure. 20% (Fr. 73–83): 12·85 cm³ 0·01 N-KOH, gef. 1·98 Mol Malonsäure.

Pentaensäure und (b) aus 10·17 mg Methylester erhaltenen Abbaudicarbonsäuren zeigen die Abb. 7 und 8. Die papierchromatographische Prüfung nach KLENK u. FAILLARD (1953) bzw. LUGG u. OVERELL (1947) ergab bei (a) für Fr. 15–40 Pimelinsäure, für Fr. 62–68 Bernsteinsäure und für Fr. 73–83 Malonsäure. Fr. 4–10 konnte nicht identifiziert werden. Bei (b) ergab die Prüfung für Fr. 3–11 nach der Verseifung Bernsteinsäure und Pimelinsäure, für Fr. 17–18 Pimelinsäure, für Fr. 58–63 Bernsteinsäure, sowie Malonsäure und für Fr. 64–84 Malonsäure.

Die reduktive Ozonidspaltung. Der Abbau von 15·2 mg Pentaensäure führte wie oben bei der Triensäure zu 2·2 mg (0·2 Mol) 2:4-Dinitrophenylhydrazon des Propionaldehyds und 7·72 mg (0·6 Mol) Hydrazon des Capronaldehyds. Der unter den Abbaualdehyden ausserdem noch angetroffene Acetaldehyd wurde nicht bestimmt. Wie im Fall der Triensäure waren die säulenchromatographisch getrennten Hydrazone papierchromatographisch einheitlich und mit den Testsubstanzen identisch.

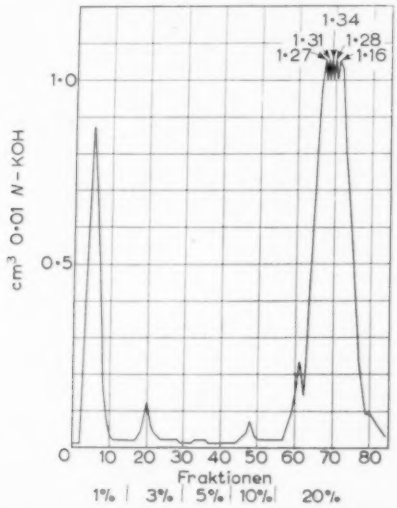

ABB. 8.—Chromatogramm der Abbausäuren des Dokosapentaen-säuremethylesters mit Säule D. 1% (Fr. 3–11): 3·08 cm³ 0·01 N-KOH + 2·4 cc 0·01 N-KOH durch Verseifung, gef. 0·82 Mol Pimelin-u. Bernsteinsäure-monomethylester. 3% (Fr. 17–28): 0·3 cm³ 0·01 N-KOH, gef. 0·05 Mol Pimelinsäure. 10% (Fr. 44–51): 0·18 cc 0·01 N-KOH, gef. 0·03 Mol nicht identifizierte Dicarbonsäure. 20% (Fr. 58–63): 0·65 cc 0·01 N-KOH, gef. 0·11 Mol Bernsteinsäure und Malonsäure. (Fr. 64–84): 12·86 cc 0·01 N-KOH, gef. 2·1 Mol Malonsäure.

DIE PAPIERCHROMATGRAPHISCHE UNTERSUCHUNG DER DOKOSAPOLYENSÄUREN

Sämtliche Versuche wurden nach der von BERNHARD (WAGNER, ABISCH u. BERNHARD, 1955) modifizierten KAUFMANNschen Methode (KAUFMANN u. NITSCH, 1954) ausgeführt, mit welcher schon früher (KLENK u. MONTAG, 1957; KLENK u. TOMUSCHAT, 1957) bei den Polyensäuren brauchbare Ergebnisse erzielt werden konnten. Zum Vergleich wurden auch die angefallenen, durch Gegenstromverteilung abgetrennten Tetraensäure (Fr. 520–560) und Hexaensäure (Fr. 250–420), sowie die noch sehr unreine und nur in sehr kleinen Mengen erhaltene Diensäurefraktion (Fr. 624–632) geprüft (Abb. 9). Die 5 Säuren sind papierchromatographisch einheitlich. Die in dem Triensäurepräparat noch als Verunreinigung vorhandene C_{24}-Tetraensäure, ist papierchromatographisch nicht nachzuweisen, da beide Säuren dieselbe Wanderungsgeschindigkeit haben. In der Diensäurefraktion bestehen die hauptsächlichsten Verunreinigungen aus den im Chromatogramm unsichtbar bleibenden Polyensäuremethylestern, die im ursprünglichen Säuregemisch noch in sehr kleinen Mengen vorhanden waren und die sich bei der CRAIG's schen Verteilung als schnellst wandernde Fraktion an dieser Stelle anreichern. Ein alle 5 Säuren in gleichen Mengen enthaltendes Gemisch ist papierchromatographisch scharf in die

Abb. 9.—Papierchromatogramm der durch Gegenstromverteilung abgetrennten C_{22}-Polyensäuren. Relative Wanderungsgeschwindigkeiten gegenüber der Tetraensäure ($R_4 = 1{\cdot}00$): Diensäure $R_4 = 0{\cdot}43$; Triensäure $R_4 = 0{\cdot}64$; Pentaensäure $R_4 = 1{\cdot}28$; Hexaensäure $R_4 = 1{\cdot}71$.

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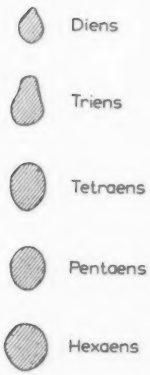
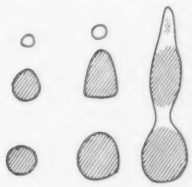


Abb. 10.—Papierchromatogramm des die abgetrennten Polyensäuren in gleichen Mengen enthaltenden künstlichen Gemischs.

Abb. 11.—Papierchromatogramm des ursprünglichen C_{22}-Polyensäuregemischs vor der Craigverteilung.



Komponenten zu trennen (Abb. 10) Führt man den Versuch mit dem ursprünglichen C_{22}-Polyensäuregemisch aus, so ist darin auf papierchromatographischen Wege das Vorhandensein der Trien-, Tetraen- und Hexaensäure einwandfrei festzustellen, nicht dagegen das Vorkommen der Pentaensäure (Abb. 11).

Anerkennung.—Die Untersuchung wurde durch Mittel der Deutschen Forschungsgemeinschaft unterstützt.

ZUSAMMENFASSUNG

Durch Gegenstromverteilung wurden aus dem C_{22}-Polyensäuregemisch der Glycerinphosphatide des Gehirns die in Mengen von 5–10% vorkommenden Triensäure und Pentaensäure herausfraktioniert. Nach dem Ergebnis des oxydativen und reduktiven Ozonidabbaus handelt es sich um $\Delta^{7,10,13}$-Dokosatriensäure, sowie um ein Gemisch von 70–80% $\Delta^{4,7,10,13,16}$- und 10–30% $\Delta^{7,10,13,16,19}$-Dokosapentaensäure.

Das ursprüngliche Gemisch der C_{22}-Polyensäuren zeigt folgende Zusammensetzung: Diensäure und nicht identifizierte Anteile 5%, Triensäure 8%, Tetraensäure 38%, Pentaensäure 6%, Hexaensäure 43%.

Näher untersucht wurde auch das papierchromatographische Verhalten der isolierten C_{22}-Polyensäuren.

SUMMARY

From the mixture of C_{22}-polyenoic acids obtained from the phosphatides of the ox brain, the trienoic and pentaenoic acids, which occur to about 5–10 per cent, were separated by counter-current partition. By the method of oxidative and reductive degradation of the ozonides it was shown that these consist of $\Delta^{7,10,13}$-docosanetrienoic acid and a mixture of 70–80 per cent $\Delta^{4,7,10,13,16}$- and 20–30 per cent $\Delta^{7,10,13,16,19}$-docosanepentaenoic acids.

The original mixture of C_{22}-polyenoic acids contained 5 per cent dienoic and unidentified acids, 8 per cent trienoic acid, 38 per cent tetraenoic acid, 6 per cent pentaenoic acids and 43 per cent hexaenoic acid.

The behaviour of the isolated C_{22}-polyenoic acids on paper chromatography was examined.

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Journal of Neurochemistry, 1958, Vol. 2, pp. 243–248. Pergamon Press Ltd., London

NATURE OF THE LINKAGE OF THE ALDEHYDE RESIDUE OF NATURAL PLASMALOGENS

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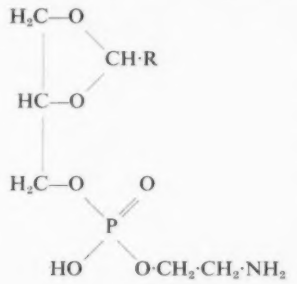
(*Received* 26 *September* 1957)

FEULGEN and VOIT (1924) first demonstrated with tissue slices that the cytoplasm gave an aldehyde reaction with fuchsin-sulphurous acid. FEULGEN and his co-workers (1929) were able to isolate the aldehydes (called plasmals, because they occurred in the plasma of the cells) and identified them as a mixture of palmital and stearal; finally, FEULGEN and BERSIN (1939) separated a phospholipid from an extract of beef heart muscle which contained these aldehydes linked as an acetal. To this acetalphospholipid (or plasmalogen, because it produced the aldehydes (plasmals) after acid treatment) they ascribed the following formula:

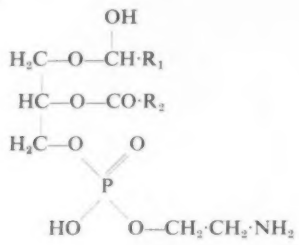


(I)

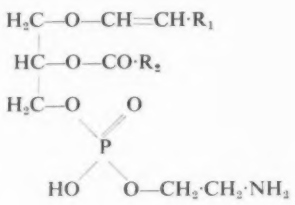
THANNHAUSER, BONCODDO and SCHMIDT (1951) isolated the same lipid in low yield from ox brain tissue. However, both authors were able to obtain this acetal-phosphatide only after treatment of the whole lipid mixture with hot alkali.

FEULGEN (1939) himself discussed the possibility of a change in the naturally occurring lipid during the alkali treatment. ANCHEL and WAELSCH (1944) observed that tissues give the fuchsin colour reaction much more quickly than a synthetic acetalphosphatide, from which they concluded that the natural product probably has a different aldehyde linkage. Furthermore, observations from KLENK's laboratory on the different kephalin fractions from human brain (KLENK and BÖHM, 1951) could not be explained on the basis of the suggested formula of the acetalphospho-lipids. In some other laboratories, too (SCHMIDT *et al.*, 1951; RAPPORT, LERNER and ALONZO, 1954), it was suspected that the natural plasmalogen must have a structure different from that proposed by FEULGEN and BERSIN, though there was no direct evidence until KLENK and DEBUCH (1954) showed that the ethanolamine-containing plasmalogen of brain contains a fatty acid as well as a fatty aldehyde in the molecule. KLENK and DEBUCH (1954) did not investigate the nature of the

linkage between the aldehyde and the glycerol, but they discussed three possible formulae.



(II)



(III)

Formula II showed the aldehyde with a hemiacetal linkage, while formula III showed the aldehyde in the enol form, giving in effect an ether bond; the aldehyde was shown in the α-position for pictorial reasons only. The authors obtained compounds similar to the phosphate esters of chimyl- or batyl-alcohols after hydrogenation and saponification of the lipid.

KLENK and GEHRMANN (1953) offered some indirect evidence for the presence of a choline-containing plasmalogen in the lecithin fraction of beef heart muscle, and HACK (1953) drew the same conclusion from experiments by paper chromatography. Early in 1955 it was shown by KLENK and DEBUCH that not only the ethanolamine–plasmalogen but also the choline–plasmalogen contains one fatty acid radical in addition to the aldehyde. After mild acid treatment of the so-called lecithin fraction of beef heart muscle, of which nearly 40 per cent consisted of choline plasmalogen and 60 per cent of lecithin, they were able to separate the unchanged lecithin from the lysolecithin produced from the plasmalogen. About 6 months later RAPPORT and ALONZO (1955) came to the same conclusion, after estimating the amount of aldehyde present by a photometric method. They called the choline plasmalogen 'phosphatidalcholine'. This term seems to be unsystematic and there is no good reason for discarding the name 'plasmalogen', given by FEULGEN. Conclusive evidence for the existence of fatty acid in ethanolamine plasmalogen was given by DEBUCH (1956) in the same way as for the choline plasmalogen a year before.

In the present paper is given the chemical proof that formula III (discussed in 1954) is correct for the natural plasmalogen, with regard to the linkage between the aldehyde residue and the glycerol.

In a compound with this formula, the double bond between the α- and β-carbon atoms of the aldehyde would be attacked by ozone. The final product after ozonization, hydrolysis and further oxidation of the enol ether of the hexadecanal would be a *n*-pentadecanoic acid, and that of the octadecanal would be a *n*-heptadecanoic acid. The ester linkage of the plasmalogen would not be affected by ozone. However, all the double bonds of the unsaturated aldehydes and acids would also be oxidized and a mixture of mono- and dicarboxylic acids formed; these split products must have a chain length of C_9 or less. Only from the saturated aldehydes would it be possible to obtain long chain fatty acids with an odd number of C-atoms.

EXPERIMENTAL AND RESULTS

(a) *Separation of a purified ethanolamine-kephalin fraction from brain.* Extraction of the brain, separation of the crude ethanolamine-kephalin fraction by FOLCH's

method (1942) and purification by counter-current partition was carried out by the method previously described (DEBUCH, 1956). A mixture of phosphatidyl ethanolamine and ethanolamine plasmalogen was obtained containing 1·9 per cent N, 3·5 per cent P, 12·0 per cent glycerol and 21 per cent plasmal (calculated as dimethylacetals), indicating that nearly 70 per cent of the total P could be ascribed to the plasmalogen. No choline and no amino acid-N could be detected. All estimations were done as described previously (DEBUCH, 1956).

(b) *Oxidation and isolation of the split products.* The purified ethanolamine kephalin fraction (2 g) was dissolved in 40 ml of chloroform and ozonized for 1 hr at −20°. (No bromine uptake could be observed.)

After addition of 15 ml of glacial acetic acid, the colourless solution was concentrated to a small volume under reduced pressure. For further oxidation and hydrolysis, the solution was brought to a total volume of 40 ml with glacial acetic acid, and 8·0 ml of H_2O_2 was added. The solution was allowed to stand in an oven at 37° for 72 hr. Then the acetic acid was distilled off through a column 40 cm high, using a good vacuum pump. The last drops of solvent were distilled off without the column by cooling the receiver to −60° and the white viscous residue was dried in a desiccator over NaOH for 2 days. For separation of the fatty acids with longer chain length from the water-soluble material, the residue was suspended in water, transferred to a separating funnel and extracted 4 times with light petroleum (b.p. under 50°). The emulsions formed were allowed to stand for several hours. The combined light petroleum solutions were washed 3 times with water to remove the water soluble material completely.

After drying the light petroleum solution over Na_2SO_4, the solvent was distilled off, leaving a white residue (0·56 g). This mixture of fatty acids was dissolved in 5 ml of acetone, cooled slowly to −40° (to remove all fatty acids of shorter chain length) and the crystallized material recovered (0·16 g Fract. A). The mother liquor was evaporated, but the residue (0·39 g Fract. B) was liquid even at room temperature. A paper chromatogram (according to KAUFMANN and NITSCH (1954) and modified by WAGNER *et al.* (1955)) of Fract. A showed that the chief fatty acids had R_F values between those of the normal C_{14} and C_{16}, and C_{16} and C_{18} fatty acids respectively. Therefore *n*-pentadecanoic and heptadecanoic acids were synthesized as reference substances.

(c) *Synthesis of* n-*pentadecanoic and* n-*heptadecanoic acids.* Myristic and palmitic acids (2·0 g of each) were the starting materials. Each sample was converted to the corresponding acid chloride, by adding 6 g of thionyl chloride, leaving for 15 min at room temperature and finally boiling under reflux for 30 min. The acid chloride was converted into the diazoketone with diazomethane and the diazoketone was oxidized with silver nitrate and ethanol to the methyl ester of the next higher homologue of the original fatty acid (ARNDT and EISTERT, 1935). After distillation, the esters were saponified and the free fatty acids obtained (m.p. 50·5° and 62·0° respectively).

(d) *Comparison between the synthetic products and those obtained by oxidation of the plasmalogens.* The mixture of fatty acids (Fract. A) obtained, was again chromatographed on paper. Commercial fatty acids (myristic, palmitic and stearic acids) as well as the synthetic fatty acids formed the reference substances.

As shown in Fig. 1, the main spots of the fatty acids obtained after ozonization had the same R_F values as the synthetic compounds. An attempt was made, therefore, to separate the different fatty acids and get pure fractions with 15 and 17

C-atoms. A chromatographic method on Hyflosupercel (according to KAPITEL, 1952) permitted the estimation of the composition of the fatty acid mixture (Fig. 2) as well as the separation of fraction differing in chain length.

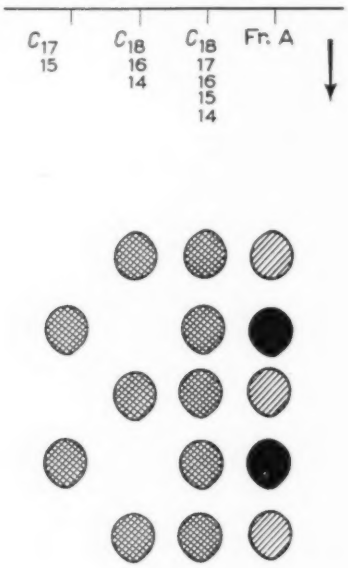


FIG. 1.

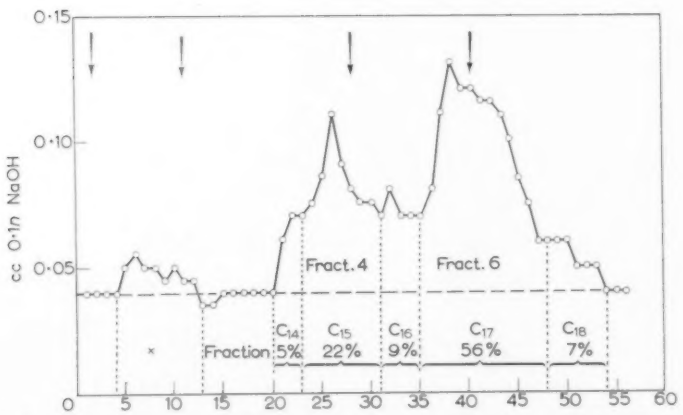


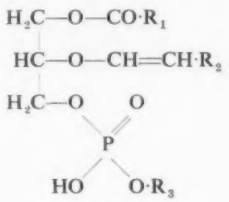
FIG. 2.—Chromatogram on Hyflosupercel of the fatty acid mixture obtained from ozonized plasmalogen shows the change of the solvent mixture X = traces of shorter chain acids.

In Fig. 3 is shown a paper chromatogram of the two pure main fractions from the Hyflosupercel column, together with the synthetic C_{15} and C_{17} fatty acids and the normal C_{14}, C_{16} and C_{18} fatty acids. The R_F values of the fractions are the same as those of the C_{15} and C_{17} compounds.

DISCUSSION

In view of the presence of nearly 80 per cent of higher fatty acids with an odd number of carbon atoms in the ozonized product, it may be presumed that the

aldehyde residues of natural plasmalogens occur in the enol form and the following formula may be given for the natural plasmalogen.



(IV)

The chemical evidence for the β-position of the aldehyde has recently been given by GRAY (1957), after RAPPORT and his co-workers (1957) had drawn the same

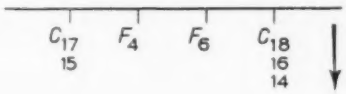


FIG. 3.

conclusion from the fact that phospholipase A attacks the choline plasmalogen. Formula IV was also given by RAPPORT, LERNER, ALONZO, and FRANZL (1957), who treated a mixture of ethanolamine plasmalogen and phosphatidyl ethanolamine from bovine muscle with alkali. They observed that the isolated acetalphosphatide, which had lost the fatty acid by this procedure, had an uptake of 0·88 mole of bromine per gram atom of phosphorus. They concluded, therefore, that there must be a double bond in the molecule and that the aldehyde must be in the enol form. But even with a recrystallized product, the uptake of bromine gives no evidence of the position of the double bond in the molecule. It seems not unlikely that unsaturated aldehyde compounds are also present in this particular plasmalogen. As LEUPOLD (1950) showed, more than 50 per cent of all the lipid aldehydes of the brain are unsaturated.

SUMMARY

An ethanolamine-kephalin fraction from human brain, purified by counter-current partition, was ozonized and split by further oxidation, yielding carboxylic

derivatives. From these, longer chain monocarboxylic acids were separated. After chromatography on Hyflosupercel, two acids were recovered as main constituents. Paper chromatography showed that those had R_F values intermediate between C_{14} and C_{16} and C_{16} and C_{18} *n*-fatty acids respectively. They correspond exactly to those of reference C_{15} and C_{17} acids obtained by synthesis. Thus, it might be concluded that the aldehydes of the plasmalogens exist in the enol form.

Acknowledgement—I wish to thank Professor E. KLENK for his continuous encouragement while this work was in progress. The technical assistance of Mrs. I. SELBACH and Miss M. L. KRAFFT is also gratefully acknowledged. The work was supported by the Deutsche Forschungsgemeinschaft.

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Journal of Neurochemistry, 1958, Vol. 2, pp. 249–253. Pergamon Press Ltd., London

THE ACTION OF RESERPINE ON THE EXCRETION OF ADRENALINE AND NORADRENALINE

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(*Received* 1 *November* 1957)

RESERPINE causes a decrease of the concentration of 5-hydroxytryptamine (5-HT) in intestine (PLETSCHER, SHORE and BRODIE, 1955) and brain (PLETSCHER, SHORE and BRODIE, 1956) as well as in all other tissues of the body in which it is normally present (PARRATT and WEST, 1957). According to SHORE, SILVER and BRODIE (1955), this action is associated with a mobilization of tissue stores rather than a defect in synthesis or increased destruction, since it is accompanied by increased excretion of 5-hydroxyindoleacetic acid, which is a major metabolite of 5-HT. Reserpine also causes a disappearance of adrenaline and of noradrenaline from the central nervous system (HOLZBAUER and VOGT, 1956), sympathetic ganglia and autonomic nerves (MUSCHOLL and VOGT, 1957*a*), but it does not cause the release of histamine (PARRATT and WEST, 1957).

It has been suggested that these effects of reserpine on 5-HT, adrenaline and noradrenaline may be the cause of some of the known effects of this drug (MUSCHOLL and VOGT, 1957*a*; BRODIE *et al.*, 1957). If the disappearance of adrenaline and noradrenaline from the tissues is due to the release of these amines, it might be accompanied, like that of 5-HT, by an increase in the excretion of the amines or their metabolic products in the urine.

The first object of the present investigation was to determine whether the administration of reserpine has any action on the rate of urinary excretion of adrenaline or of noradrenaline in man. The second object was to compare data collected from patients known to be sensitive to reserpine with those collected from patients known to be resistant.

METHODS

The patients used in these experiments were all chronic male schizophrenics. At the beginning of the experiment each patient was given an injection of the drug vehicle at 0900 hours, and the urine collected over a period of 24 hr. Preliminary experiments confirmed the observation that the rate of excretion of catecholamines during the day is greater than the rate of excretion during the night (VON EULER *et al.*, 1955; VON EULER, 1956). The urine was therefore collected in two samples, from 0900 to 1700 hours, and from 1700 hours to 0900 hours the following day. (The only deviation was in the case of patient number 1. In this case the urine collection was from 0900 to 1300 hours.) After a variable but continuous number of such collections, reserpine was given by intramuscular injection in place of the injection of vehicle, and the urine collected as usual. The dose was 15 mg per patient, except for case 1, who received 10 mg.

Free urinary adrenaline and noradrenaline were determined fluorimetrically after absorption on IRC-50 and separation by paper chromatography, as described by CRAWFORD and LAW (1958).

The patients were divided into two groups. Group A had all been previously treated with reserpine, but had not received any drugs for at least 2 weeks prior to the experiment, and no reserpine for

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at least 3 weeks. It was known therefore which patients tended to be resistant to reserpine. This classification, based on prolonged therapy, was confirmed by observation of the effects of a single injection. The patients classified as susceptible all showed at least three symptoms normally associated with reserpine. These symptoms included drowsiness, restlessness, nausea and vomiting, flushing, abdominal cramps and bowel movement. The patients classified as resistant showed not more than one of these symptoms.

Group B had been receiving continuous reserpine therapy (10–15 mg a day by mouth) for a minimum of 4 weeks, when the first sample was analysed. Reserpine therapy was then discontinued in these three patients for 24 days and the urine samples were again collected and analysed.

RESULTS

The results obtained in these experiments appear in Table 1. A single administration of reserpine was followed by a marked fall in the rate of excretion of adrenaline and of noradrenaline. These falls occurred in every case except one, and were clearly significant. They were seen both in patients classified as susceptible and in those classified as resistant, and there was no clear difference between the effects in these two groups.

Chronic treatment with reserpine resulted in a severely diminished rate of excretion of adrenaline and of noradrenaline, as indicated by the fact that after the administration of reserpine was discontinued, the rates of excretion rose within 24 days to values similar to those found in other patients before treatment.

TABLE 1.—RATE OF EXCRETION OF ADRENALINE AND NORADRENALINE IN THE URINE OF SCHIZOPHRENIC PATIENTS (μmg/min)

	Adrenaline			Noradrenaline			Volume of urine		
	Control	Reserpine	%	Control	Reserpine	%	Control	Reserpine	%
A. Patients susceptible to reserpine									
1	42	14	33	38	13	34	778	323	42
2	30	8·3	28	27	14·7	54	1,050	440	42
3	21	10	48	10	7·2	72	1,200	595	50
4	24	12	50	33	2·5	8	1,085	520	48
Patients resistant to reserpine									
5	54	9·3	17	35	25	71	1,000	830	83
6	18	18	100	26	22	85	903	532	59
7	24	11	46	38	13	34	1,120	754	67
B. Patients receiving prolonged treatment									
8	23	7·5	33	70	3·3	5	725	475	65
9	53	7·5	14	35	3·8	11	780	470	60
10	72	3·2	4	32	2·5	8	1,130	350	31

These large doses of reserpine also caused an antidiuretic effect and a fall of blood pressure. The volume of urine excreted during the first day after a single dose of reserpine fell by about 44 per cent. This fall was a striking feature in each case and was quantitatively similar to the percentage fall in the rate of excretion of adrenaline and noradrenaline. There was no significant change in the concentrations of the amines in the urine after a single dose of reserpine. On the other hand, prolonged treatment with reserpine in the last three cases had a greater effect on the excretion of the two amines and there was a significant fall of 50–93 per cent in their concentrations in the urine.

The change in the volume of the urine was not due to changed drinking habits as the fluid intake was kept reasonably constant for each patient, and the mean estimated fluid intake was kept constant within 1 per cent. Fluid loss from sweating and defaecation may account in part for the diminished urine output, but there was probably a true fluid retention.

The mean systolic blood pressure fell on the first day after a single dose of reserpine by an average of 13 mm of mercury (S.D. 3·5, $P < 0{\cdot}02$). On the second day it was still low, but on the third day it had risen to approximately the control value.

DISCUSSION

The control values for the rate of adrenaline excretion are much larger than those given by VON EULER (1956) for normal patients; the values for noradrenaline are about the same. According to VON EULER the ratio of the amount of noradrenaline to that of adrenaline in human urine is normally about 5; in these data it was about 1. This difference may be due to the use of different techniques, or to the excretion by schizophrenic patients of increased amounts of adrenaline. We have at present no figures for the rate of excretion in normal persons estimated by the method used here.

The antidiuretic effect of reserpine has been previously observed in rats (GAUNT, RENZI, ANTONCHAK, MILLER and GILMAN, 1954), dogs (GOULD and SHERROD, 1957) and man (KROGSGAARD, 1956) and explained in various ways. MARLEY and PARE (1956) obtained other evidence for fluid retention in man. LANGEMANN and GOERRE (1957) saw no change in the volume of urine in man after reserpine, but they used comparatively small doses. In the present experiments the effect was seen after a single large dose and seemed to be a fairly sensitive index of the action of the drug.

The results shown in the table are calculated in terms of the weight of free amine excreted per minute. This is the usual method of presenting the results and is supported by the observations of KÄRKI (1956), who found no correlation between the rate of excretion of these amines by humans and the volume of urine. However, since a single dose of reserpine had no significant action on the concentration of the amines in the urine, it might perhaps be suggested that its observed action on the rate of excretion in these experiments was secondary to its antidiuretic action. This explanation would not apply to the results, in group B, where repeated doses of reserpine depressed both the rate of excretion of the amines and their concentration.

Taken as a whole, the results indicate that reserpine caused a fall in the rate of liberation of both adrenaline and noradrenaline, instead of the rise which had been expected on the analogy of the evidence regarding 5-HT. This might be regarded as confirmation of the results of BURGER (1957), who obtained evidence of a fall of the

concentration of noradrenaline in human plasma after reserpine. There is however, some doubt about the methods of estimation which he used (Holzbauer and Vogt, 1954).

There was no clear difference between the effect of reserpine on the excretion in those patients classified clinically as susceptible to reserpine, and those classified as resistant. This may mean that the mobilization of these two amines is not associated with the clinical effect. On the other hand, the amount mobilized in the brain is likely to be small compared with the total amount mobilized in the body and thus to have little effect on the amount measured in the urine. It is reasonable to suppose that reserpine did mobilize the amines in the brain and that this effect was not detected by the tests made on the urine. It is more likely that the change in the excretion of catecholamines is secondary, and that the urine provides a more sensitive method of detecting sedation than that provided by the clinical observations made in these patients. A few observations of the rate of excretion of catecholamines during the night indicated that the rate of excretion during the day after reserpine was similar to the rate of excretion during the night in the control period. On the other hand, the very low rates of excretion after prolonged treatment may well be due to a direct action of reserpine on the formation or fate of catecholamines.

The failure to detect increased excretion of the amines after reserpine does not prove that different mechanisms are involved in the actions of this drug on 5-HT and sympathin. Muscholl and Vogt (1957*b*) have reviewed the published evidence that reserpine causes various effects which might be due to an initial release of sympathomimetic amines and have demonstrated a rise in the adrenaline content of rabbits' blood during the first hour after an injection of reserpine. It is possible that both adrenaline and noradrenaline are released from stores in the human, thus finding their way to blood and urine, but that the quantities are too small to be detected by the methods used here. Because of the long periods of collection of the urine, the effect of a brief period of increased excretion might be obscured by its summation with the effect of a long period of diminished excretion. Similar difficulties have arisen in connection with 5-HT. For example, Langemann and Goerre (1957) found that the main effect of reserpine on the rate of excretion of 5-hydroxyindoleacetic acid in normal man was a fall, sometimes preceded by a small brief rise; patients with carcinoid tumours showed a larger rise.

SUMMARY

The rate of excretion of adrenaline and noradrenaline in the urine of schizophrenic patients during the day was estimated fluorimetrically after chromatographic separation of the amines.

The mean figure for noradrenaline was similar to that obtained previously in normal patients by other methods, but the mean figure for adrenaline was higher.

Reserpine (15 mg) depressed the rate of excretion of both amines. It also had an antidiuretic action, but the result probably indicates a true fall in the rate of liberation of both amines in the body, which is presumably a secondary effect of the sedation.

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Journal of Neurochemistry, 1958, Vol. 2, pp. 254–260. Pergamon Press Ltd., London

A COLLAGEN-LIKE COMPOUND ISOLATED FROM BOVINE SPINAL CORD—I

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(*Received* 24 *June* 1957)

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IN recent years, interest has been focused on the chemistry of the central nervous system. The available information reflects a rather uneven picture, because the lipids have been studied in great detail, whereas the proteins, which constitute about 30 to 40 per cent of the solid material, according to MCILWAIN (1955), are described rather as ill-defined fractions, instead of well characterized compounds. Exceptions to this statement are the conjugated proteins isolated by FOLCH and co-workers, FOLCH and LEES (1951), LEBARON and FOLCH (1956), PORTER and FOLCH (1957).

In our effort to isolate the allergic encephalomyelitic agent from nerve tissue, bovine spinal cord was extracted with various organic solvents and the extracts and the residue examined for activity. It was found that the lipid-free residue which contains the proteins is highly active (KIES, ROBOZ and ALVORD, 1956). Therefore, this residue was further investigated. Preliminary tests indicated that one of the active fractions was a collagen-like compound, referred to as C.L. protein. This report describes the isolation, purification and characterization of this protein. Studies on its biological activity are presented in Part II.

EXPERIMENTAL

Isolation procedure. Bovine spinal cord† was removed immediately after slaughtering the animal, the meninges removed, the cord partially extracted with acetone and benzene, and dried. The pre-extracted spinal cord was defatted by successive extractions in a Soxhlet apparatus with the following solvents: acetone, ether, chloroform, mixture of chloroform-methanol and ethanol. The lipid-free residue was extracted with water by autoclaving it at 15 lb pressure for 8 hr. The suspension was filtered while still warm, and the extract was dialysed and lyophilized. The dialysed fraction was further purified by cold fractionation with ammonium sulphate. The following fractions were obtained:

Fraction I: 28 per cent saturation, turbidity
Fraction II: 36 per cent saturation, slight precipitate
Fraction III: 60 per cent saturation, large flocculent precipitate
Fraction IV: 90 per cent saturation, small turbidity

From these fractions, only number III was used for further purification. It was dialysed at 4° until sulphate was completely removed. The lyophilized protein was redissolved in water and a small

* This work was supported in part by grants from the U.S. Public Health Service and the Multiple Sclerosis Society.
† Kindly supplied by Armour and Company Research Laboratories, Kankakee, Illinois.

insoluble residue removed in a refrigerated centrifuge. The clear supernatant was reprecipitated at 55 per cent saturation, dialysed at 4°, and lyophilized.

Analytical methods. Total N* was determined by Dumas' method, after the sample was dried to constant weight.

The isoelectric point was determined by precipitation with the detergent, sodium dodecyl-sulphate, according to the method of PUTNAM and NEURATH (1944).

Hydrolysis of the protein. The protein (84 mg) was hydrolysed with 4 ml of 6 N-HCl in a heavy-walled glass stoppered centrifuge tube. The tube was tightly closed with a steel clamp and heated at 104° for 24 hr. For the determination of tryptophan, 83 mg of the protein were hydrolysed with 5 ml of 3 N-NaOH.

The circular paper chromatographic technique of SAIFER and ORESKES (1956) was used for the separation of the amino acids with a solvent mixture of *n*-butanol, acetic acid and water (40 : 10 : 50 v/v). Two-dimensional chromatograms were also prepared according to the method of IRREVERRE and MARTIN (1954). The distribution of the amino acids was also studied by paper electrophoresis with phthalate buffer pH 5·8 and acetic acid of pH 2·3 according to DURRUM (1951).

Amino acids with the exception of hydroxyproline and hydroxylysine were quantitatively determined by microbiological assay procedures (H. M. Chemical Co., 1956; WRIGHT, 1952). The procedure for alanine did not give satisfactory results unless a liver preparation† was added to the basal medium. In the tryosine determination, hydrogen peroxide-treated peptone was added to the regular media in a ratio of 75 ml to 500 ml. *Leuconostoc mesenteroides P*-60 was used instead of *Lactobacillus casei* for the tyrosine bio-assay. Since hydroxyproline and hydroxylysine cannot be assayed by a microbiological procedure, the first was determined by the chemical procedure of MARTIN and AXELROD (1953), and the latter by a quantitative paper chromatographic technique of DUBUISSEAU *et al.* (1957).‡

For the identification of the carbohydrates conjugated with the protein, 100 mg of the compound were hydrolysed with 5 ml 2 N-HCl in a heavy glass-walled centrifuge tube, as previously described, and the hydrolysate concentrated *in vacuo*. In order to obtain well defined spots on the chromatogram, the amino acids were removed by treatment with Amberlite MB-2. For the best separation of the sugars, a mixture of butanol, pyride, benzene and water in a ratio of 5:3:1:3 was used with multiple development, as described by DEWHALLEY, ALBAN and GROSS (1951) and ROBOZ *et al.* (1955). After the sugars were identified, known amounts of reference carbohydrates were used together with the hydrolysate for quantitative estimation. For the determination of the total hexose, the procedure of ROBOZ, *et al.* (1955) was used. For the determination of hexosamine, BLIX's (1948) modification of the method of ELSON and MORGAN was used.

RESULTS

Preparation and properties of the C.L. protein

The yield of collagen-like protein from cord acetone powder is 3·2 per cent. This is equivalent to a 1·6 per cent yield from whole lyophilized cord. The protein is water-soluble and can be precipitated with cold ethanol, trichloroacetic acid and phosphotungstic acid. The nitrogen content is 17·61 per cent. Neither sulphate nor phosphate is present.

Ultracentrifugal and electrophoretic measurements on the C.L. protein carried out by Dr. M. MACKENZIE and reported elsewhere indicated a homogeneous compound. All electrophoretic experiments revealed essentially one component with slight variations in the shape of the curves. The mobilities of the collagen-like protein at the various pH levels indicated an isoelectric point at approximately pH 4·6. Structural-type proteins may likewise exhibit a similar behaviour in the electric field. Thus, elastin has a nearly constant mobility from pH 4·5 to pH 6·5 (PARTRIDGE,

* Microanalyses were made by Oakwold Laboratories, Alexandria, Va.

† The preparation used was Lilly's Reticulogen, U.S.P.

‡ We are indebted to Dr. DUBUISSEAU of the National Cancer Institute for the hydroxylysine determination.

DAVIS and ADAIR, 1955). Skin collagen (to which the collagen-like protein is very similar in amino acid composition) has an isoelectric point of 4·3, according to THEIS and JACOBY (1941).

From the diffusion and sedimentation constants, the calculated molecular weight of the collagen-like protein under the conditions of analysis is approximately 38,000. This compares with a value of 52,000 obtained in one light scattering experiment.

TABLE 1.—MOLECULAR WEIGHT OF C.L. PROTEIN BASED ON AMINO ACID COMPOSITION

	(1) Wt. per cent	(2) Mol. wt.	(3) Residue wt.	(4) G Moles per 100 g protein	(5) Assumed number of residues	(6) Wt. of residues	(7) Min. mol. wt.	(8) Apparent min. mol. wt.
Glycine	20·45	75	57	0·272	109	15·50	366	39,670
Alanine	8·2	89	71	0·092	37	6·5	1085	40,145
Leucine	4·8	131	113	0·036	14	4·06	2730	38,220
*iso*Leucine	2·3	131	113	0·017	7	1·92	5695	39,865
Valine	3·4	117	99	0·029	12	2·87	3441	41,292
Phenylalanine	2·8	165	147	0·016	6	2·35	5893	35,357
Tyrosine	1·01	181	163	0·006	2	0·98	17921	35,842
Tryptophan	0·0	—	—	—	—	—	—	—
Serine	3·25	105	87	0·030	12	2·61	3231	38,772
Threonine	2·9	119	101	0·024	10	2·42	4103	41,030
Cystine	0·0	—	—	—	—	—	—	—
Methionine	0·7	149	131	0·005	2	0·65	21285	42,570
Proline	12·6	115	97	0·109	44	10·57	912	40,128
Hydroxyproline	11·9	131	113	0·090	36	10·17	1101	39,636
Lysine	5·4	146	128	0·037	15	4·73	2703	40,545
Hydroxylysine	1·3	161	143	0·008	3	1·14	12384	37,152
Arginine	8·2	174	156	0·047	19	7·33	2122	40,318
Histidine	1·8	155	137	0·011	4	1·50	8611	34,444
Aspartic Acid	5·5	133	115	0·041	16	4·71	2420	38,720
Glutamic Acid	11·5	147	129	0·078	31	10·06	1278	39,618
Total:	108·01			0·948	379	90·07	Average:	39,046

Electron micrographs of the collagen-like protein showed extremely long fibrils. A detailed description of the foregoing macromolecular characteristics of this material is given in the report of Dr. MACKENZIE (1957). The specific rotation was $[\alpha]_D^{20} = -106°$, in 4 per cent aqueous solution at 20°.

The ultraviolet spectrum as determined in a Beckman DU spectrophotometer showed no absorption peak at 280 mμ. According to NEURATH and BAILEY (1954), the ultraviolet spectrum of collagen is unlike other proteins, because collagen contains so few side chains with aromatic rings. The lack of absorption at 280 mμ has been suggested by LOOFBOUROW and associates (1949) as a means of assessing the purity of collagen preparations.

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The isoelectric point of pH 4·5, determined by precipitation with sodium dodecylsulphate, agrees with the value of 4·6 obtained from mobility measurements.

Amino acid composition. The protein hydrolysate was studied qualitatively by paper chromatography and paper electrophoresis. The presence of hydroxyproline and hydroxylysine was established by their colour reaction with isatin and ninhydrin as well as their R_F values.

TABLE 2.—AMINO ACID COMPOSITION OF COLLAGENS*

	Spinal cord†	Cattle bone‡	Ox bone§	Human tendon§
Glycine	20·5	23·3	25·3	25·4
Alanine	8·2	—	10·5	10·3
Leucine	4·8	3·4	3·9	3·6
*iso*Leucine	2·3	2·1	1·7	1·5
Valine	3·4	2·7	2·7	3·1
Phenylalanine	2·8	2·6	2·9	2·5
Tyrosine	1·01	0·98	0·56	0·69
Tryptophan	0·0	0·01	—	—
Serine	3·3	3·1	4·2	4·1
Threonine	2·9	2·3	2·5	2·3
Cystine	0·0	0·11	—	—
Methionine	0·7	0·72	0·80	0·90
Proline	12·6	—	14·7	15·2
Hydroxyproline	11·9‖	—	14·1	12·6
Lysine	5·4	5·5	4·1	3·3
Hydroxylysine	1·3¶	—	1·1	1·5
Arginine	8·2	9·0	9·2	8·9
Histidine	1·8	0·64	0·96	0·87
Aspartic acid	5·5	—	7·1	6·7
Glutamic acid	11·5	11·2	11·9	11·1

* All figures expressed as per cent dry weight.
† Microbiological methods, present authors.
‡ Microbiological methods, NEUMAN (35).
§ Resin column chromatography, EASTOE (33).
‖ Chemical method.
¶ Chromatographic method.

The amino acid composition presented in Table 1 served as a basis for the calculation of the mean residue weight and minimum molecular weight, according to the method of BERGMANN (1935, 1936, 1937); see also CHIBNALL (1942). The mean residue weight of 95 compares favourably with values of 92·6 and 93·7 reported for collagen from other sources (BOWES and KENTEN, 1948). ASTBURY (1940), using X-ray data and the density of dry gelatin, obtained a value of 94. The apparent minimum molecular weight calculated from columns 5 and 7 of Table 2 gave an average of 39,046. From the mean residue weight, the calculated minimum molecular weight was 36,000. BOWES and KENTEN (1948) reported a similar molecular weight for ox-hide collagen of 38,730.

A comparison of the amino acid composition of this protein with collagen from other sources is presented in Table 2. The composition is similar to that of the other

collagens. It is rich in glutamic acid, proline, hydroxyproline and glycine, although the concentration of the latter amino acid is lower than in the other collagens. The high hydroxyproline content (11·9 per cent) and the presence of hydroxylysine (1·3 per cent) are accepted characteristics of collagen.

Carbohydrates. Analysis for carbohydrates yielded a value of 1·45 per cent for hexoses and 0·22 per cent for hexosamine. The monosaccharides present in the hydrolysate were identified by paper chromatography as galactose, mannose, glucose and fucose. Quantitative estimation by the use of reference sugars in various concentrations was carried out. Galactose, mannose, glucose and fucose were found in the relative amounts of 10 : 5 : 4 : 1. The presence of galactose, mannose and glucose has been reported in cow-hide collagen by GROSS, HIGHBERGER and SCHMITT (1952). The hexosamine content of ox-hide collagen was found to be 0·33 per cent (BOWES, ELLIOTT and MOSS, 1953).

DISCUSSION

The presence of collagen in the central nervous system has been indicated by reports based on quantitative determination of collagen by LOWRY, GILLIGAN and KATERSKY (1941) and LOGAN, MANNELL and ROSSITER (1955). Collagen, however, has not been previously isolated from nervous tissue.

The amino acid composition of the bovine cord protein is very close to that of collagen from other sources, with the exception of glycine (20·5 per cent). Collagen from skin has 23–26 per cent glycine. A fibrous protein was isolated recently by MATOLTSY (1952) from the vitreous humor of the eye and its amino composition studied by GROSS, MATOLTSY and COHEN (1955). This protein, which the authors called vitrosin, and classified as a member of the collagen class, contained 19 per cent glycine, a value close to the one reported here. Detailed comparative studies would be needed to establish whether the two proteins were related.

The carbohydrates in the C.L. protein are non-dialysable. The possibility that a polysaccharide is present as a contaminant of the protein was considered. However, since ammonium sulphate precipitation was used in the purification process, and this reagent does not precipitate polysaccharides unless they are firmly bound to the protein, one must consider the carbohydrate as an integral part of the protein molecule.

Another protein which is similar to collagen and is widespread in mammalian tissues is elastin. Therefore, it was also considered that the cord protein might belong in this group. If comparison is made on the basis of the amino acid composition, this protein shows a closer similarity to collagen than to elastin. The best evidence that this compound is derived from collagen is its high hydroxyproline content and the presence of hydroxylysine.

The molecular weight, as calculated from the amino acid composition, agrees with the results of BOWES and KENTEN (1948). The results obtained by sedimentation and light scattering were 38,000 and 52,000 respectively. This low molecular weight indicated that the collagen, as expected, was modified by heat treatment. The molecular weight for native collagen, as established by BOWES and MOSS (1951), is very high. These authors found no terminal amino groups by the use of SANGER's dinitrofluorobenzene method. They interpreted their findings as indicating that the molecular weight of collagen is several million.

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The presence of ground substance in nervous tissue has been indicated by the histochemical methods of BAIRATI (1953) and HESS (1953). Recently, GLEGG and PEARCE (1956) showed the presence of an acid mucopolysaccharide containing 26·9 per cent uronic acid and 15·9 per cent hexosamine. It is suggested that the collagen-like protein isolated from spinal cord may be associated with an acid mucopolysaccharide, and that they together form the ground substance of the nervous tissue as they do in other tissues. As to the source of the collagen in the central nervous system, one can only speculate whether it is derived from the nerve tissue proper or from the small blood vessels which form an integral part of the total structure.

SUMMARY

A fibrous protein has been isolated from bovine spinal cord and purified. It was found to be homogeneous by the criteria of electrophoretic and ultracentrifugal measurements.

Its physical properties, including isoelectric point, optical rotation, and molecular weight, have been studied. The amino acid composition was determined by paper chromatography, paper electrophoresis and microbiological assay. The carbohydrate content has been determined, and the individual sugars identified as glucose, mannose, galactose and fucose.

The amino acid composition has been compared with that of collagen from other sources and found to be similar. High hydroxyproline content and the presence of hydroxylysine suggest that the compounds belongs to the collagen group; therefore, it has been designated as a collagen-like compound.

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THE ALLERGIC ENCEPHALOMYELITIC ACTIVITY OF A COLLAGEN-LIKE COMPOUND FROM BOVINE SPINAL CORD—II

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(*Received* 24 *June* 1957)

THE isolation of a purified protein from nervous tissue exhibiting encephalitogenic activity is of considerable importance because of the opportunity offered for definitive studies on the disease mechanism. Heretofore, immunological studies on this intriguing biological reaction have been hampered by the unavailability of a soluble antigen. Early work showed that a lipid-soluble fraction from bovine spinal cord contained some encephalomyelitic activity when tested in rabbits (WAKSMAN *et al.*, 1954), mice (OLITSKY and TAL, 1952) and guinea pigs (GOLDSTEIN *et al.*, 1953), but the 'active' fractions were water-insoluble and thus not suitable for immunological tests.

The experimental results presented here show that the major portion of the encephalitogenic activity of bovine spinal cord occurs in the lipid-free residue, and that part of this activity is associated with the C.L. protein described in Part I.

EXPERIMENTAL

As previously described by KIES *et al.* (1957), adult male guinea pigs were maintained in a closed colony at the National Institutes of Health. Following an initial observation for 2 weeks, groups of 10 animals were injected intradermally or subcutaneously with emulsions containing various amounts of cord or cord extracts in Freund's complete adjuvant. The animals were observed at regular intervals for the development of clinical signs of the experimental disease. After 30 days they were anaesthetized, blood collected, and the animals killed by perfusion intracardially with neutral buffered 10% formalin. All experimental animals were assigned a disease index of 0 to 10 depending on the severity of the clinical and histologic reactions and the presence of lipemia.† Clinical and histologic evaluations were made by independent observers. Ten guinea pigs were used for each level of antigen tested and the average disease index for each group was plotted against the dose injected on a logarithmic scale. Since the maximum disease reaction resulted in a disease index of around 8, a half maximal reaction (50 per cent effective dose) was defined as 4. For purposes of calculating specific activity (units/mg) or total activity of each fraction, the amount of material eliciting a disease index of 4 was said to contain one unit of allergic encephalomyelitic activity. The details of this method will be published elsewhere (ALVORD and KIES, 1957).

* This work was supported in part by grants from the U.S. Public Health Service and the Multiple Sclerosis Society.

† It has been shown that the presence of a marked lipemia is associated with the more severe stages of the disease in guinea pigs (KIES *et al.*, 1957).

RESULTS

Disease index data for all of the cord fractions studied are shown in Figs. 1 and 2. ED-50 values from these curves were used in the calculation of the specific activity of each fraction listed in Table 1.

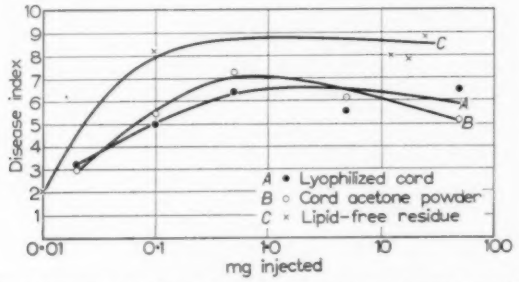

FIG. 1.—Encephalitogenic activity of lyophilized bovine cord, partially defatted cord, and lipid-free residue.

From the curves in Fig. 1 it will be noted that large amounts of whole cord and partially defatted cord appear to be inhibitory. The lipid-free fractions have not shown this behaviour. The maximum disease index values for whole cord and cord acetone powder were 6·6 and 7·3, whereas the maximum values for all of the other fractions varied from 7·9 to 9·0.

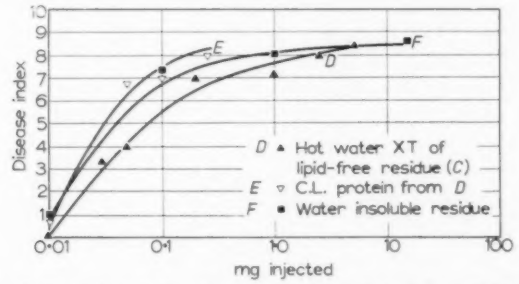

FIG. 2.—Encephalitogenic activity of protein fractions derived from lipid-free residue of bovine cord.

In Table 1 are listed specific activities, as well as total activities, of the various cord fractions. The specific activity indicates the extent of purification of a fraction. The total activity is a measure of the stability and/or recovery of the activity originally present in whole tissue. In addition to the cord fractions shown in Figs. 1 and 2, four lipid preparations derived from bovine cord or bovine brain white matter are listed in the table. The specific activity of these lipid fractions is much lower than any of the protein fractions studied.

DISCUSSION

The availability of a quantitative assay is a major prerequisite for the successful isolation of a biologically active compound. Lack of a good quantitative test resulted in misleading interpretations of much of the early work on allergic encephalomyelitis, including our own preliminary report on the activity of the water-soluble material from cord acetone powder. Injection of 0·2 mg of the soluble protein caused a higher

percentage of severe clinical reactions than 50 mg of either crude preparation (KIES, ROBOZ, and ALVORD, 1956).

In order to establish quantitative relationships between fractions, repeated tests were made at different levels of all the fractions studied. When enough tests had been made, it became clear that whole cord and partially defatted cord showed inhibition

TABLE 1.—ENCEPHALITOGENIC ACTIVITY OF BOVINE CORD FRACTIONS

	Specific activity* (Units/mg. dry wt.)	Weight (%)	Total activity† (Units)	(%)
(A) Lyophilized cord	18	100	1,800	100
(B) Cord acetone powder	22	50	1,100	61
Ether extract	0·1	8	—	<0·1
$CHCl_3$—CH_3OH extract	0·04	5	—	<0·1
(C) Lipid-free residue	45	27	1,200	67
(D) Hot water extract of (C)	20	4·4	90	5
(E) C.L. protein from (D)	40	1·6	60	3
(F) Residue from (D)	33	21·6	700	39
(G) Bovine white matter				
Total lipid extract (Folch)	<0·1	—	—	—
Proteolipid (A and B)	<0·1	—	—	—

* One unit = 50 per cent effective dose.
† Weight of fraction obtained from 100 mg whole tissue multiplied by the specific activity of the fraction.

when injected in large amounts (over 1 mg). Thus the comparison between 50 mg cord acetone powder and 0·2 mg glycoprotein fraction was not a valid one. Each fraction had to be tested repeatedly until the concentration range below maximal activity for that fraction was established. Although maximum activity was never obtained with whole cord or partially defatted cord, lower levels yielded a dose-response curve similar to those of other cord fractions. The results given in Table 1 were all based on comparable activity ranges.

From the data in Table 1, it can be seen that the most important purification was achieved in the removal of lipids from partially defatted cord (compare B with C). At this stage of the fractionation there was no apparent loss in activity, even though a small amount of activity was detected in the lipid extracts. (The assay is not sufficiently quantitative to differentiate small differences in total activity, even though it is sensitive enough to detect extremely small amounts of activity.) In view of the fact that a sample of the whole cord from which the cord acetone powder was prepared was not available for assay, it is impossible to determine whether or not the difference between A and B total activities is significant.

It is evident that the major portion of the encephalitogenic activity in bovine spinal cord is associated with the non-lipid portion. This conclusion is based on tests with guinea pigs and may be a characteristic of the species, since other workers have reported that the lipid fractions of cord contain most of the encephalitogenic activity when tested in rabbits or mice.

The extraction of the lipid-free residue with hot water under pressure resulted in considerable loss of activity. (Fractions D and F account for only 65 per cent of the activity in Fraction C.) Whether this is due to destruction caused by the drastic

extraction conditions or whether it is a decrease in activity resulting from separation of two or more active constituents needs further investigation. It is noteworthy that Residue F still contained 39 per cent of the total activity after it had been subjected repeatedly to the same extraction procedure which yielded Fraction D.

The increase in specific activity of the C.L. protein over that of the partially defatted cord was much less than the extent of chemical purification. Since the C.L. protein appears to be pure by the criteria used (ultracentrifugation and electrophoresis) the activity may reside in a compound present in such small quantities that it was not detectable by these techniques. Another possibility is that several antigens are present in the whole tissue, some of which have been removed during fractionation. (The lipid antigen reported by other workers cannot explain these changes in activity during fractionation, because the activity of the lipid extract is almost negligible when tested by this technique in the guinea pig.) A third possibility, which is under investigation at the present time, is that native collagen prepared from unheated and non-solvent extracted nerve tissue might possess greater activity than the modified form which was isolated.

There have been conflicting reports on the antigenicity of collagen. WATSON and ROTHBARD (1954) have demonstrated a complement-fixing antibody in rabbit serum after injection of collagen from rat tail tendon. Whether the ability to form antibodies is related to encephalitogenic activity is an open question, since a definite correlation has never been established between these two phenomena, both of which occur as a result of whole brain injections. The availability of a water-soluble protein antigen should facilitate investigation of this question.

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SUMMARY

Encephalitogenic activity has been demonstrated in a purified collagen-like protein isolated from bovine spinal cord. Quantitative comparison of this compound with whole lyophilized cord indicated a two- to threefold increase in specific activity. In comparison to the extent of chemical purification this increase in specific activity was not sufficient to warrant the conclusion that this compound represented the main encephalitogenic agent in nervous tissue. It has been suggested that traces of a more active protein are adsorbed on the purified C.L. protein or that other antigens have been removed during fractionation. A third possibility is that the activity of C.L. protein has been attenuated by the isolation procedures used.

The encephalitogenic activity of lipid fractions from bovine cord or bovine brain white matter is almost negligible compared to the activity in the lipid-free residue when tested in the guinea pig.

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Journal of Neurochemistry, 1958, Vol. 2, pp. 265–282. Pergamon Press Ltd., London

CONCENTRATION OF SOME ANALGESIC COMPOUNDS AND THEIR ANALOGUES IN TISSUES SURVIVING *IN VITRO:* RELATION TO *IN VITRO* METABOLISM AND *IN VIVO* EFFECTS

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(*Received* 27 *September* 1957)

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IN order to assess accurately the metabolic effects produced in tissues *in vitro* by analgesic and other agents, it is necessary to determine the concentration of these compounds in the tissues involved. This aspect of metabolic studies has been little investigated (MILLER and ELLIOTT, 1954; MCILWAIN, THOMAS and BELL, 1956). In the present work the assimilation of some analgesic agents and related compounds in tissues *in vitro* and factors influencing this have been determined. An attempt has been made to correlate these concentrations with observed effects *in vitro* and *in vivo*.

Morphine, *p-cyclo*hexyloxy-α-phenylethylamine (CHP), which is an analgesic when injected (i.p.) at a dose level of 10–20 mg/kg into albino rats, and *p-iso*propyloxy-α-phenylethylamine (IPP), which although very similar in structure to CHP is non-analgesic at dose levels of 40 mg/kg (MCCOUBREY, 1953), were the main compounds used in this study. The *in vivo* distribution of CHP and IPP has been studied by BRIERLEY and MCCOUBREY (1953), and these drugs were shown to reach concentrations in the brain many times those present in the blood stream. The concentration of morphine in the brain, during analgesic action, however, has been shown to be small (MILLER and ELLIOTT, 1955). It was thought that these drugs would form an interesting comparison.

EXPERIMENTAL

Materials

Drugs. *p-cyclo*hexyloxy-α-phenylethylamine (CHP), an analgesic; *p-iso*propyloxy-α-phenylethylamine (IPP), inactive analgesically; *p-cyclo*hexyloxy-α-phenylethylallylamine (CHPA), an analgesic antagonist; and *p-cyclo*hexyloxybenzoic acid (CHB) were prepared and supplied by A. MCCOUBREY. In experiments dealing with their accumulation by tissues α^{14}C-labelled drugs were used, the position of the labelling being marked by an asterisk in the diagram of their structures (Fig. 1). Their activities were respectively 30,211 or 8,286, 10,559, 26,246 and 35,088 counts/min/mg. Morphine and N-allyl normorphine (Fig. 1) were used as the hydrochlorides.

The drugs were dissolved in Krebs–Ringer saline (KREBS and HENSELEIT, 1932) containing neither glucose nor calcium salts, at such a concentration that addition of 0·35 ml to the 3·15 ml of saline in the vessels produced the desired final concentration (except where otherwise stated in the text).

Inhibitors. Sodium iodoacetate and potassium cyanide were dissolved in saline, as above, such that addition of 0·1 ml to the saline in the vessels produced the desired final concentration.

Salines. In most experiments Krebs–Ringer phosphate saline was used, buffered at suitable pH, usually 7·4. In some experiments the buffer of the above saline was replaced by glycylglycine (final concentration 25 mM) or aminotrishydroxymethylmethane (final concentration 50 mM) or bicarbonate. The pH of the bicarbonate saline was varied by using either different concentrations of sodium bicarbonate or a 30% carbon dioxide/70% nitrogen gas mixture, in place of the more usual 5%/95% mixture.

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Substrates. Succinic, L-glutamic, α-oxoglutaric, pyruvic (20 mM) or lactic acids (33 mM) were adjusted to pH 7·2 with NaOH; lactic acid was boiled with glass-distilled water and pyruvic acid distilled before neutralizing. Glucose was used at a final concentration of 10 mM.

Procedure

Preparation of tissues. Adult albino rats (about 300 g) were stunned by a blow on the back of the neck, bled, the brain removed, and slices cut and weighed as described by MCILWAIN (1951). The slices were either transferred intact to conical manometric vessels or trimmed into rectangles before

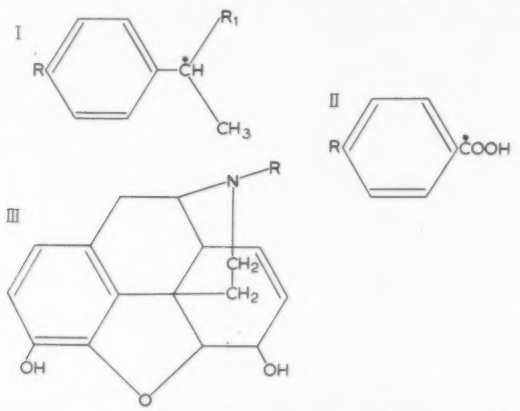

FIG. 1.—Structures of substances examined. *p-cyclo*Hexyloxy-α-phenylethylamine: I, R = OC_6H_{11}, R_1 = NH_2. *p-iso*Propyloxy-α-phenylethylamine I, R = OC_3H_7, R_1 = NH_2. *p-cyclo*Hexyloxy-α-phenylethylallylamine: I, R = OC_6H_{11}, R_1 = NHC_3H_5. *p-cyclo*-Hexyloxy-benzoic acid: II, R = OC_6H_{11}. Morphine: III, R = CH_3. *N*-Allylnormorphine: III, R = C_3H_5

weighing and floating on to silver grid electrodes, type *H*, in vessels type *A* of approximately 10 ml capacity (AYRES and MCILWAIN, 1953). Suspensions were prepared from excised cerebral cortex with a glass test tube homogenizer, in Krebs–Ringer phosphate or bicarbonate saline. Homogenization was for approximately 3 min.

In some experiments blocks of cerebral cortex and diencephalon were removed, weighed, and chopped into prisms, 0·35 × 0·35 × 2 mm, by means of a mechanical chopper (MCILWAIN and BUDDLE, 1953). The chopped blocks of tissue were transferred into electrode vessels type *F* with gold ring electrodes and of 4 ml capacity (AYRES and MCILWAIN, 1953); 1 ml of Krebs–Ringer saline and 10–20 mg of tissue (wet weight) were used in these vessels.

Hearts were excised while still beating and placed on to saline-moistened filter paper until the blood had been pumped out. Slices were then rapidly cut from the ventricles with a razor blade and glass guide. The diaphragms of young rats of about 100 g were dissected out and the peripheral muscular portions used.

Electrical stimulation. Electrical stimulation was by condenser pulses (MCILWAIN, 1951) supplied at a frequency of 100/sec, time constant 0·3–0·4 msec and a peak voltage of 8 to 12 v.

Manometric measurements. Respiration was measured manometrically in the salines mentioned before (except bicarbonate-containing solutions) in an atmosphere of O_2. The vessels were placed in the bath at 37·5° approximately 25 min after the death of the animal. Experiments were usually of 90 min duration; pulses, when applied, were either used throughout the experimental period or only in the second 30-min period. Anaerobic experiments were either carried out in phosphate buffered saline under an atmosphere of N_2 or in bicarbonate-buffered saline under an atmosphere of 5% CO_2/95% N_2 or 30% CO_2/70% N_2. Sticks of yellow phosphorus were used in the centre wells of the vessels.

Chemical analysis. Lactic acid was determined by the method of BARKER and SUMMERSON (1941) on aliquots of fluid removed from the vessels at the end of the experimental period.

For analysis of morphine or *N*-allylnormorphine tissue was homogenized in 5% trichloroacetic acid (TCA) and centrifuged at high speed. Aliquots of the supernatant were taken, neutralized, and made up to a standard volume for assay. The method of analysis involved mixing 3 ml of TCA extract

of tissue or 3 ml of a mixture of solution from the manometric vessels and TCA with 0·25 ml of undiluted Folin and Ciocalteau reagent, leaving for 10 min at room temperature, adding 0·75 ml of 25% Na_2CO_3, incubating for 30 min at 37° and reading the resultant colour at 760 mμ in a Hilger Uvispectrophotometer. I am indebted to R. RODNIGHT for the details of the above method.

Radioactive technique. The technique used was as follows:

Radioactive drug in Krebs–Ringer saline was pipetted into the side arm of the vessels; after incubating the flasks for 10 min at 37·5° the drug was tipped into the main compartment of the vessel and incubated with the tissue for the times stated in the text. The slices were then rapidly removed, surplus moisture blotted off on a glass plate, and transferred to a weighed amount of kieselguhr on a tared watch glass. An aliquot of fluid from the vessel was also removed and pipetted into a weighed beaker containing a weighed amount of kieselguhr. The samples were dried overnight at 60°, weighed, intimately mixed and 30 mg of the resultant powder assayed at 'infinite' thickness (POPJAK, 1950) under a G.E.C. EHM2 mica window (1·9 mg/cm²) counter. The counting efficiency by comparison with a standard ^{14}C polythene disc was 1·9 per cent and a linear relation between dilution of samples with kieselguhr and activity was found in trial assays. 2–3000 counts were registered for each sample. Background count (12–15 counts/min) was determined on samples of fluid and tissue, treated as above, from vessels without added drug.

Experiments to determine the concentration of drugs by suspension were carried out in test tubes. Cerebral cortex suspension (3·15 ml) was transferred by means of a wide bore pipette into a test tube containing 0·35 ml drug. The contents of the tube were thoroughly mixed and the tube placed in a constant temperature bath and shaken throughout the experimental period. The tube was then removed and the contents rapidly centrifuged at 0°, the fluid was decanted off from the tissue debris and the tube was further drained by resting its lip on filter paper. The tissue debris was washed with distilled water into a weighed beaker containing a weighed amount of kieselguhr. An aliquot of the fluid, decanted as above, was pipetted into a similar beaker containing kieselguhr. Radioactivity was assayed as before.

When the tissue was incubated with radioactive drug in Krebs–Ringer saline buffered with bicarbonate, the alkalinity developing on drying overnight portions of the drug in salines, appeared to facilitate loss of the drug, possibly by destruction. This loss could be prevented by the addition of concentrated hydrochloric acid.

The concentration ratio which is equal to mg drug in 1 g moist tissue/mg drug in 1 ml fluid was calculated as shown below:

$$x = \text{mg drug in 1 g moist cortex} = \frac{(\text{counts/min of sample} - \text{counts/min for background}) \times \text{wt. of dried sample} \times k)}{30 \text{ (i.e. wt. of dried sample assayed)} \times \text{moist wt. of slice}}$$

$$y = \text{mg drug in 1 ml fluid} = \frac{(\text{counts/min of sample} - \text{counts/min for background}) \times \text{wt. of dried sample} \times k}{30 \text{ (i.e. wt. of dried sample assayed)} \times \text{ml of fluid taken} \times 1{,}000}$$

Concentration ratio $= x/y$

k is a constant and is equal to mg of drug which gives 1000 counts/min.

RESULTS

In the present series of experiments with glucose as substrate and in the absence of applied electrical pulses, rat cerebral cortex slices respired at approximately 76 μmoles O_2/g moist weight/hr and the lactic acid formed during incubation for 90 min was about 39 μmoles/g moist weight. Applied electrical pulses, of a suitable type, increased these values to 122 μmoles O_2/g moist weight/hr and 66 μmoles/g moist weight respectively.

Actions of the drugs on tissue metabolism

Effects of drugs used on the glucose metabolism of cerebral cortex slices in the absence of pulses. CHP, IPP, and CHPA produced either a progressive inhibition of

respiration or were without effect for about 30 min, followed by an abrupt decrease in oxygen uptake. Fig. 2 shows typical experiments.

Effects on respiration began at 2×10^{-4}M with CHP, 4×10^{-3}M with IPP, and

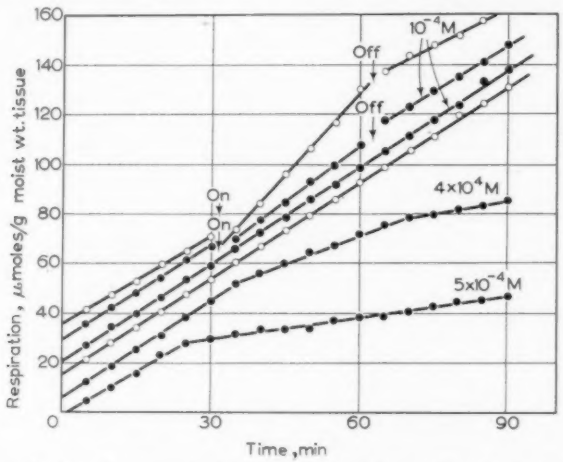

FIG. 2.—Respiration of rat cerebral cortex slices (≏50 mg) in 3·5 ml phosphate-buffered Krebs–Ringer saline, with added CHP (●) and without added drug (○). The concentration of drug used (M) is shown on the graph. The tissue was in grid electrodes, type *H*, in vessels type *A*, and the drug, where present was in the saline before addition of the tissue. Electrical pulses, peak potential 8v, were applied in two cases, as shown by the arrows. Ordinates are displaced for clarity.

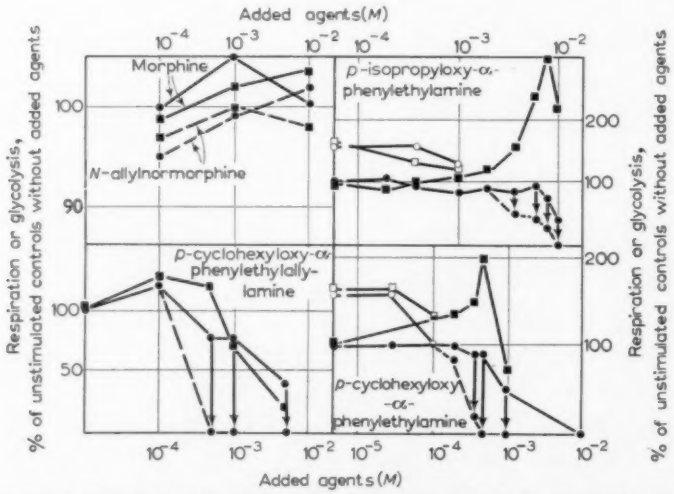

FIG. 3.—Metabolism of rat cerebral cortex slices, conditions as in Fig. 2. Respiration with (○) and without (●), pulses and concomitant formation of lactic acid with (□) and without (■) pulses (peak potential 8v). The arrows run from points giving the results during the first 30 min of metabolism to points giving the results during the second 30 min. All points give the average result of 4 to 6 experiments.

4×10^{-4}M with CHPA (Fig. 3). Morphine or *N*-allylnormorphine at concentrations between 10^{-2}M and 10^{-4}M were without effect on the oxygen uptake of rat cerebral cortex slices (Fig. 3). The latter results confirm those of SEEVERS and SHIDEMAN (1941).

Beginning at approximately 10^{-4}M an increased lactic acid production was seen in the presence of CHP. At a concentration of 5×10^{-4}M, the lactic acid production had increased by 100 per cent over the rate in control slices without added drugs. Stronger concentrations of drug produced a fall in the production of lactic acid (Fig. 3). Similar effects were seen with IPP, the non-analgesic compound, at stronger concentrations of drug (Fig. 3). CHPA did not cause such a marked increase in lactic acid production as the above two drugs (Fig. 3). Lactic acid production in the absence of drug was $38 \pm 1{\cdot}9$ μmoles/g moist weight and in the presence of drug at a concentration of 10^{-4}M, $52{\cdot}6 \pm 3{\cdot}1$ μmoles/g moist weight.

Morphine and *N*-allylnormorphine at concentrations of 10^{-2}M, 10^{-3}M and 10^{-4}M were without effect on lactic acid production of cerebral cortex slices (Fig. 3).

Greater effect of α-phenylethylamines on cerebral cortex slices in the presence of applied pulses and the effect of potassium salts. The effect of applied pulses on the respiration of rat cerebral cortex slices in silver grid electrodes, type *H*, in vessels type *A* (AYRES and MCILWAIN, 1953), and the result of added drug are shown in Fig. 2, which gives a typical experiment.

Application of pulses resulted in an 80% (approx.) increase in respiration, while CHP (10^{-4}M) abolished this response (Figs. 2 and 3). This concentration of drug was without effect on unstimulated metabolism. At higher concentrations of drug similar effects were seen with IPP.

Increased concentration of potassium salts in Krebs–Ringer medium also leads to increased respiration (ASHFORD and DIXON, 1935). Thus, in the present experiments, when the potassium chloride concentration of the medium was increased from $5{\cdot}1 \times 10^{-3}$M to $3{\cdot}5 \times 10^{-2}$M respiration was approximately doubled. In experiments in which the excess potassium chloride was added together to the slices with CHP (4×10^{-4}M) at the beginning of the experimental period, inhibition of respiration by the analgesic was delayed and very slightly reduced. No effect of excess potassium chloride was seen when it was tipped from the side arm of the vessel after tissue respiration had been inhibited by the drug (Table 1). JOWETT and QUASTEL (1937) have observed the same type of effect with chloretone.

Irreversible nature of the respiratory inhibitions produced by p-cyclo*hexyloxy-α-phenylethylamine and* p-iso*propyloxy-α-phenylethylamine.* The reversibility of the inhibitions produced by CHP and IPP were investigated, as shown in Table 2. No indication of a reversal of the inhibitions produced by these two drugs was obtained (Table 2). Some fall in respiration was noticed on transferring control slices, without added drug, to fresh salines. Comparable effects on the part of other drugs have been noted by other workers (QUASTEL and WHEATLEY, 1934).

Sensitivity with different substrates. Table 3 shows that with the exception of succinate, CHP (4×10^{-4}M) inhibited the oxidation of all substrates used in a similar way. With succinate as substrate (as also with glutamate and α-oxoglutarate), the the initial high respiratory rate was not maintained throughout the 90 min experimental period, and with succinate, addition of CHP (4×10^{-4}M) was without effect on the fall of respiratory rate (Table 3).

The effects of p-cyclo*hexyloxy-α-phenylethylamine,* p-iso*propyloxy-α-phenylethylamine and morphine hydrochloride on the metabolism of chopped diencephalon and cerebral cortex.* There is much evidence to suggest that the diencephalon plays a part in analgesic mechanisms (see, for example, PENFIELD, 1947; WOLFF, 1947), although

TABLE 1.—EFFECT OF POTASSIUM CHLORIDE ON THE RESPIRATORY INHIBITION OF CEREBRAL CORTEX SLICES BY *p-cyclo*HEXYLOXY-α-PHENYLETHYLAMINE

Potassium chloride added (M)	Drug	No. of observations	Respiratory rate (μmoles/g wet wt./hr)		Inhibition of respiration (%)
			Initial	Final	
$3{\cdot}5 \times 10^{-2}$	+	3	83	15	82
$3{\cdot}5 \times 10^{-2}$	+	4	132	50	62
$3{\cdot}5 \times 10^{-2}$	−	2	138	—	—
$5{\cdot}1 \times 10^{-3}$	−	1	78	78	—

Rat cerebral cortex slices (≏50 mg) were in Krebs–Ringer phosphate buffered saline under an atmosphere of O_2. The potassium chloride concentration of the above saline was $5{\cdot}1 \times 10^{-3}$M. Extra potassium chloride to give a final concentration of $3{\cdot}3 \times 10^{-2}$M was either added to the saline before the addition of the slice, or tipped from the side arm into the main compartment after tissue respiration had been inhibited by the drug, *p-cyclo*hexyloxy-α-phenylethylamine (4×10^{-4}M), the drug being present before the addition of the slice. The latter case is indicated by an asterisk in the table. The initial respiration refers to respiration during the first 30 min of incubation and the final respiration during the first 30 min of incubation and the final respiration to that in the last 30 min. The percentage inhibition of respiration refers to the respiration in the final 30 min period as a percentage of that during the initial 30 min period.

TABLE 2.—IRREVERSIBILITY OF THE INHIBITION OF RESPIRATION PRODUCED BY *p-cyclo*HEXYLOXY-AND *p-iso*PROPYLOXY-α-PHENYLETHYLAMINE

Section A

Drug	Respiratory rate before washing	Respiratory rate after washing	No. of obs.
−	$79{\cdot}5 \pm 3{\cdot}1$	$60{\cdot}0 \pm 4{\cdot}8$	4
+	$73{\cdot}2 \pm 4{\cdot}0 \rightarrow 20{\cdot}6 \pm 1{\cdot}7$	$12{\cdot}4 \pm 3{\cdot}0$	5
−	81		1
+	$78{\cdot}0 \pm 2{\cdot}0 \rightarrow 12{\cdot}0 \pm 1{\cdot}8$		4

Section B

Drug	Respiratory rate before washing	Respiratory rate after washing	No. of. obs.
—	$76 \pm 2{\cdot}2$	61 ± 3	4
+	$77 \pm 4{\cdot}1 \rightarrow 30 \pm 1{\cdot}3$	$19 \pm 2{\cdot}6$	4
−	79·0		2
+	$81{\cdot}0 \rightarrow 28{\cdot}0$		2

Slices of rat cerebral cortex were incubated with the drugs until respiration was significantly inhibited (≏40 min). They were then washed quickly in warm saline and transferred to vessels containing warm, drug-free Krebs–Ringer phosphate medium and oxygen uptake followed manometrically for a further 40 min. Experiments in section A were with *p-cyclo*hexyloxy-α-phenylethylamine (4×10^{-4}M) and in section B with *p-iso*propyloxy-α-phenylethylamine (4×10^{-3}M). Respiratory rates are given as μmoles O_2/g moist weight/hr followed by the S.E.M. Where two respiratory results are given joined by an arrow, the first rate is for the initial period of metabolism before inhibition occurs and the second gives the value for respiration just before the slices were transferred to fresh vessels, or in the case of slices which were not washed, respiration during the final 30 min period.

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TABLE 3.—INHIBITION OF RESPIRATION OF RAT CEREBRAL CORTEX SLICES BY *p-cyclo*HEXYLOXY-α-PHENYLETHYLAMINE IN THE PRESENCE OF VARIOUS SUBSTRATES.

Substrate	No. of obs.	Drug	Respiratory rates μmoles/g wet wt./hr		
			1st 30 min	2nd 30 min	3rd 30 min
Lactic acid	4	+	102	84	27
Lactic acid	4	−	99	99	99
Pyruvic acid	4	+	87	20	12
Pyruvic acid	4	−	84	84	84
α-oxoglutaric acid	4	+	76	14	0
α-oxoglutaric acid	4	−	75	50	48
Glutamic acid	4	+	96	36	0
Glutamic acid	4	−	90	76	72
Succinic acid	6	+	91	65	65
Succinic acid	6	−	96	66	65
Glucose		+	83	48	8
Glucose		−	80	80	80

Rat cerebral cortex slices were in Krebs–Ringer phosphate saline in an atmosphere of oxygen. The drug where present was at a concentration of 4×10^{-4}M in the medium before the tissue was added. All substrates were adjusted to pH 7·2 with NaOH. Results are quoted as mean values of μmoles O_2/g moist weight/hr for the number of experiments stated.

MASSERMAN (1939) reported that morphine caused little or no change in the sensitivity of the hypothalamus to stimulation. The effects of the above compounds on the metabolism of chopped cerebral cortex and diencephalon were therefore investigated, to see whether there was any difference in the response of these two tissues to the drugs. The opportunity was also taken to observe whether these two tissues responded in a similar manner to electrical stimulation.

Using chopped tissue and applying pulses at 100/sec, time constant 0·35 msec and varying the voltage between 8 and 12 v, the responses of diencephalon and cerebral cortex to the applied pulses were identical. In both tissues, respiration was increased by about 100 per cent with a current of the above characteristics and a peak potential of 7·5 v. In the present series of experiments and in the absence of applied agents diencephalon respired at about 65 and cerebral cortex at about 71 μmoles O_2/g moist weight/hr.

Varying concentrations of drug affected the respiratory and glycolytic response of cerebral cortex and diencephalon to pulses in a similar way; Fig. 4 shows this. Thus, morphine hydrochloride, at a concentration of 10^{-3}M reduced the metabolic response to pulses of both tissues by approximately one half, while 10^{-2}M reduced it still further. Fig. 3 shows that these concentrations of drug are without effect on the metabolism of cerebral cortex slices in the absence of pulses, and Fig. 4 shows that they are without effect on the respiration of chopped diencephalon, while causing a slight increase in lactic acid production. Thus, with 10^{-3}M drug, lactic acid accumulation is $67 \pm 3{\cdot}2$ μmoles/g moist weight, while in the absence of drug it is $53 \pm 3{\cdot}0$ μmoles/g, moist weight. Fig. 4 shows that CHP and IPP also affect the metabolism of cerebral cortex and diencephalon in parallel.

Concentration of drugs by tissues in vitro

This series of experiments was undertaken to determine whether metabolic effects of the drugs were produced at concentrations in the tissues *in vitro*, comparable to

those in the brain *in vivo* during physiological action. It has been shown above that CHP affects cerebral slice metabolism *in vitro* at a concentration of 4×10^{-4}M. It can be calculated from the results of BRIERLEY and MCCOUBREY (1953) that during analgesic action the concentration of this drug in cerebral cortex is $4{\cdot}2 \times 10^{-4}$M. This cannot be taken as evidence that metabolic effects are produced by the drugs at physiological concentrations without experiments to determine the extent of the concentration of these drugs by cerebral cortex slices *in vitro*.

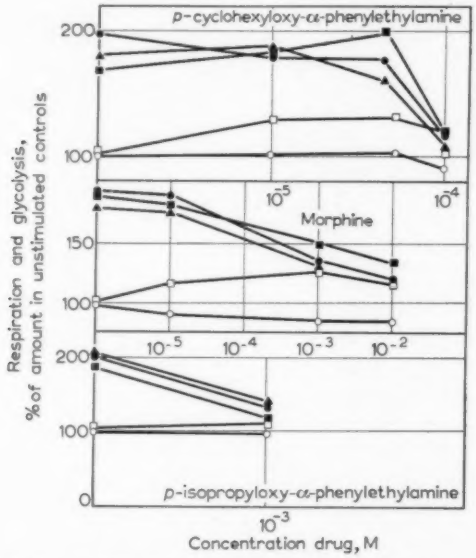

FIG. 4.—Metabolism of fragments of chopped diencephalon and cerebral cortex (≃20 mg) from rat. The tissue was in 1 ml Krebs–Ringer glucose saline in gold ring electrode vessels, type *F*. Respiration of diencephalon in the presence (●) and absence (○), and lactic acid formation in the presence (■) and absence (□) of applied pulses (peak potential 8v) and respiration of cerebral cortex with applied pulses (▲).

Accumulation of α-phenylethylamines by cerebral cortex slices in vitro. In Fig. 5 the ratio of mg drug in 1 g wet slice to mg of drug in 1 ml fluid is plotted against time. It can be seen from the graphs that the entry of the drugs was not a rapid process and no plateaus were reached on the concentration curves. Thus, with CHP (10^{-5}M) the rate of entry of the drug fell off slowly and experiments with a 120 min incubation period showed that drug was still entering the tissue between the 90 and 120 min time intervals, although at a slower rate. It was also found that the lower the concentration of drug added to the slice the higher was the concentration ratio established. For example with CHP and a 30 min incubation period, a concentration ratio of 19·2 was established with 4×10^{-4}M drug, 22 with 10^{-4}M, 26 with 4×10^{-5}M and 28 with 10^{-5}M.

Fig. 5 shows that CHP, the analgesic compound, was taken up by the slice to a far greater extent than IPP which is inactive analgesically. When these drugs were added to the saline surrounding the slice at a concentration of 10^{-5}M, a concentration ratio of 43 was established with the former and 7·6 with the latter. This difference was not detected *in vivo* (BRIERLEY and MCCOUBREY, 1953), both drugs being taken up by

cerebral cortex and the other tissues examined in approximately equal amounts.

CHPA was concentrated by cerebral cortex slices to a greater extent than CHP. The shape of the concentration curves, however, were similar. CHB, which is similar in structure to CHP, but without an amino group and of an acidic nature, is accumulated by the tissues to a much smaller extent than the analgesic, which is basic (Fig. 5).

Given in Fig. 5 are the concentrations of drug in μmoles/g moist weight reached in the tissue at the final time. It can be calculated from the results of BRIERLEY and

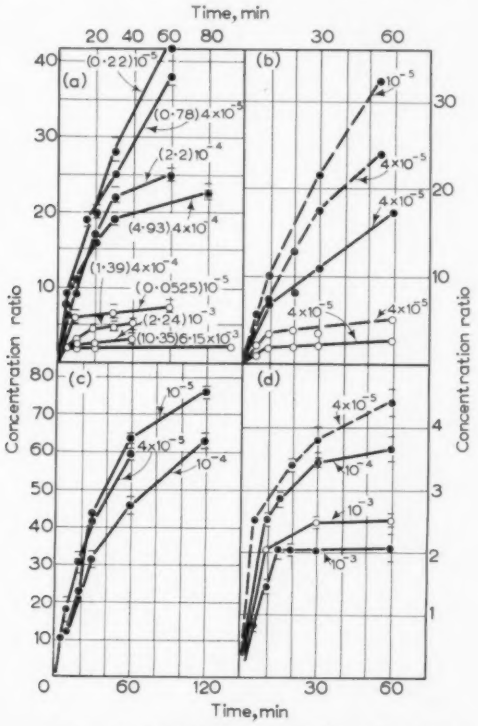

FIG. 5.—(a) The accumulation of CHP (●) and IPP (○) by cerebral cortex slices and (b) by heart (points joined by broken line) and diaphragm (unbroken line). (c) The accumulation of CHPA and (d) CHB (broken line), morphine (●) and *N*-allylnormorphine (○) by cerebral cortex slices. The tissue (55–70 mg) was in 3·5 ml Krebs–Ringer phosphate saline and the drugs were tipped into the manometric vessels from the side arm. Shown on the graph are the concentration of drug (M) added to the vessels and in parenthesis the concentration of drug (μmoles/g moist wt.) reached in the slice at the time shown. Where applicable the S.E. is given as a bar each side of the point.

MCCOUBREY (1953) that a concentration of 0·18 μmoles/g moist weight is reached in the cerebral cortex of rats when analgesia results from the injection of CHP. Applied electrical pulses had no major effect on the accumulation of drug by the slice. Thus, with CHP (8×10^{-5} M) and an incubation period of 30 min, the concentration ratio in the presence of applied pulses was $20{\cdot}2 \pm 1{\cdot}1$ (8 experiments) and in their absence $23{\cdot}2 \pm 0{\cdot}78$ (8 experiments). Excess potassium chloride in the media similarly had little effect.

Accumulation of morphine and N*-allylnormorphine by cerebral cortex slices* in vitro. Fig. 5 shows that the concentration of morphine by cerebral slices differed in two

respects from the concentration of CHP. First, the concentration curves reached stable plateaus: thus with 10^{-3}M-morphine, the accumulation of the drug by the tissue was maximal at 15 min and thereafter remained approximately constant. Secondly, high concentration ratios were not established: for example, with 10^{-4}M morphine a concentration ratio of 3·5 was established at the 60 min time interval and a ratio of 25 with 10^{-4}M CHP.

N-allylnormorphine (10^{-3}M) was concentrated by the tissue to a slightly greater extent than morphine (10^{-3}M) and this difference is significant as is shown by the standard errors plotted on the curve (Fig. 5). This effect was also observed with CHP and its antagonist (see above).

Uptake of p-cyclo*hexyloxy-α-phenylethylamine and* p-iso*propyloxy-α-phenylethylamine by heart and diaphragm* in vitro. Because of the difference observed above between the uptake of these two drugs by cerebral cortex slices and because this effect was not observed *in vivo* (BRIERLEY and MCCOUBREY, 1953), the uptake of these two drugs was followed in two other tissues to determine whether the difference was specific to cerebral cortex. Fig. 5 shows the results obtained. The concentration curves took the same form as those observed with cerebral cortex, although smaller concentration ratios were found, but, as with cerebral cortex, CHP was concentrated to a larger extent than IPP.

The concentration of the drugs used did not affect the respiration of the tissues. In the presence of the drugs, diaphragm respired at 68·3 $\pm$ 1·27 μmoles O_2/g moist weight/hr and heart at 71·8 $\pm$ 2·7 μmoles/g moist weight/hr.

Effects of various buffers on the concentration of some drugs by cerebral cortex slices. CHP was concentrated to an equal extent by cerebral cortex slices whether they were suspended in phosphate, glycylglycine or aminotrishydroxymethylmethane-buffered saline. If the slice was suspended in bicarbonate-buffered saline, however, the concentration of drug taken up by the slice was greater than that observed with the above buffers. Similar effects were seen with IPP, CHPA and morphine hydrochloride and in diaphragm as well as cerebral cortex slices. It, therefore, appeared to be a relatively unspecific effect. To test whether the results are due to variations in pH, cerebral cortex slices were suspended in Krebs–Ringer saline buffered with either bicarbonate or phosphate buffer at various pH values. Fig. 6 shows the results obtained. It appears that as the alkalinity of the medium is increased the uptake of drug by cerebral cortex slices increases. In these experiments the pH of the medium was tested with a pH-meter at the beginning and the end of the experimental period. It was found that when Krebs–Ringer saline buffered at pH 7·4 with phosphate buffer was used, the final pH after an experimental period of 60 min was around 6·8, whereas bicarbonate-buffered saline maintained its pH at about 7·4. The greater uptake of drug by slices suspended in bicarbonate buffered saline was therefore assumed to be due to the fact that bicarbonate buffer maintains a higher pH than phosphate buffer.

Experiments to determine whether the accumulation of drug is dependent on an energy source

Effect of anaerobic conditions and/or no added substrate. If the uptake of drug depended on an energy source then the lack of oxygen or substrate should considerably reduce its accumulation.

Anaerobic conditions, with added substrate, were found to reduce the concentration

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ratios established by CHP (4×10^{-5}M) by approximately one-eighth. Aerobic conditions in the absence of substrate had about the same effect, while with both anaerobic conditions and absence of substrate the ratios were reduced slightly further (Fig. 7). Even with the latter conditions, however, considerable accumulation of drug by the slice still occurred. Accumulation of morphine hydrochloride by cerebral cortex slices was not significantly affected by omitting substrate and oxygen.

Effect of inhibitors. 2:4-Dinitrophenol (4×10^{-5}M) which is an inhibitor of oxidative phosphosylation (CASE and MCILWAIN, 1951), iodoacetic acid, an inhibitor of enzymes containing SH-groups (ADLER, EULER and GUNTHER, 1938) and potassium cyanide, an inhibitor of cytochrome oxidase (STOTZ, ALTSCHUL and HOGNESS, 1938) were without effect on the concentration ratios established by CHP ($4{\cdot}5 \times 10^{-5}$M). Results are shown in Table 4.

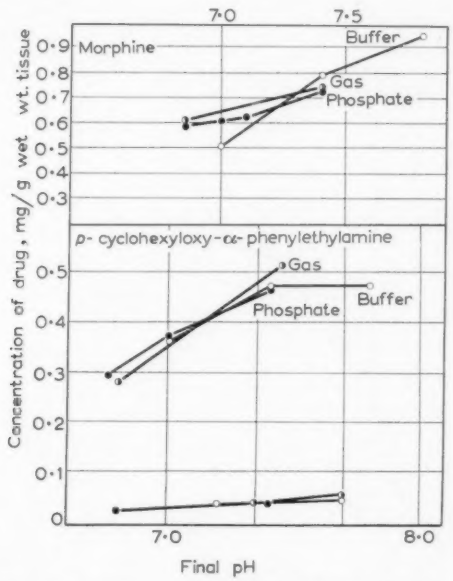

FIG. 6.—Effect of pH on the accumulation of CHP and morphine by rat cerebral cortex slices *in vitro*. The pH of the bicarbonate buffer was varied by altering the concentration of bicarbonate (indicated by the word 'buffer' on the graph) or by varying the composition of the gas phase (indicated by the word 'gas'). 'Phosphate' indicates the use of phosphate buffered saline. Incubation was for 15 min before and 30 min after addition of drug.

Sensitivity with different metabolic substrates. The effect of varying the substrate of the incubation medium was tried because it is known that substrates such as succinate and glutamate do not maintain the energy-rich phosphates of the tissue (MCILWAIN, 1952; KRATZING, 1953).

With CHP (4×10^{-5}M) as the added drug, and glucose, glutamate, pyruvate or succinate as substrates, concentration ratios of 25 to 32 were obtained after a 30 min incubation period. The accumulation of drug by cerebral cortex slices appeared to be slightly greater in incubation media containing 20 mM of either pyruvate, glutamate or succinate as substrate than in those containing glucose. Thus, with succinate as substrate, concentration ratios of $32{\cdot}4 \pm 0{\cdot}76$ were obtained, with glutamate

27·4 ± 2·04 with pyruvate 31·8 ± 0·88, and glucose 27·4 ± 0·51, 25 ± 0·6 and 26 ± 1·5 respectively in parallel experiments with the former substrates.

Experiments were also tried on the effect of substituting glutamate (20 mM) for glucose (10 mM) on the accumulation of morphine hydrochloride (10^{-3}M) by cerebral cortex slices. After an incubation period of 15 min the concentration ratios in the presence of glutamate or glucose were identical. With a longer incubation period (30 min) the concentration ratio with glucose as substrate was 2·1 ± 0·1 and with glutamate 2·8 ± 0·3. It appears likely that these small differences in drug uptake may be due to small differences in alkalinity.

Accumulation of p-cyclo*hexyloxy-α-phenylethylamine by cerebral cortex suspensions.* Suspensions of rat cerebral cortex were prepared as described before, such that the ratio of tissue to fluid was equivalent to that in tissue slice experiments. After 30 min incubation with CHP (4×10^{-5}M), a suspension in Krebs–Ringer phosphate buffered saline gave a concentration ratio of 27·5 (3 experiments) and after 60 min incubation,

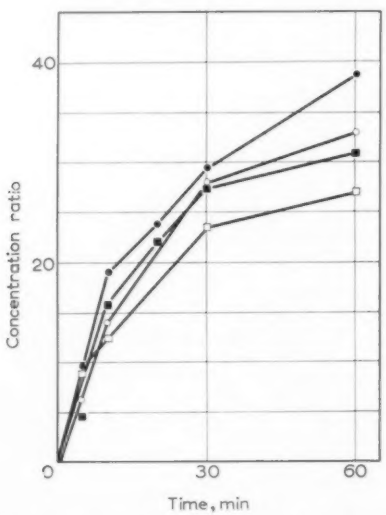

FIG. 7.—Effect of anaerobic conditions in the presence of glucose (○), aerobic conditions in the absence of substrate (■), anaerobic conditions without added substrate (□) and aerobic conditions in the presence of glucose (●) on the accumulation of CHP (4×10^{-5}M) by rat cerebral cortex slices *in vitro*.

32·5 (6 experiments). Comparable values in slices are 26·0 and 38·0 respectively. With five times as much tissue in the same volume of saline, cerebral cortex suspensions gave concentration ratios of about 43·8 after 60 min incubation.

Removal of accumulated drug in the slice by washing. Table 5 shows that IPP, which is inactive analgesically, was more easily washed from the slice than CHP. Thus, after incubating tissue for 40 min with 2×10^{-4}M-IPP or 6×10^{-5}M-CHP, when the concentration of these drugs in the tissue were approximately equal, 77 per cent of the former and 39 per cent of the latter would be washed from the slice.

Similar results were obtained with bicarbonate-buffed saline and CHP (4×10^{-5} M) and IPP (10^{-4}M) when 45·1 per cent of the former and 89·4 per cent of the latter could be washed from the slice.

TABLE 4.—ABSENCE OF EFFECT OF SOME INHIBITORS ON THE ACCUMULATION OF *p-cyclo*HEXYLOXY-α-PHENYLETHYLAMINE

	Incubation time (min)	Inhibitor	Concentration ratio	No. of expts	Respiration (μmoles/g moist wt./hr
(I)	2:4-DNP ($4{\cdot}5 \times 10^{-5}$M)				
	30	+	$31 \pm 2{\cdot}8$	6	$161{\cdot}2 \pm 3$
	30	−	$26 \pm 2{\cdot}0$	5	$77{\cdot}5 \pm 2{\cdot}3$
(II)	Iodoacetic acid (10^{-4}M)				
	30	+	32	2	71 → 66·6
	60	+	39	2	
	30	−	33·1	2	76
	60	−	41·4	2	
(III)	Iodoacetic acid (6×10^{-4}M)				
	30	+	28	3	54 → 14
	30	−	32	3	85
(IV)	Potassium cyanide (10^{-4}M)				
	30	+	26	2	70 → 62
	60	+	42	2	
	30	−	27·2	2	77
	60	−	41·5	2	
(V)	Potassium cyanide (10^{-3}M)				
	30	+	28	2	32
	60	+	37·5	2	
	30	−	29	1	78
	60	−	40	2	

Slices of cerebral cortex (50–60 mg) were in Krebs–Ringer, phosphate buffered, glucose saline (3·5 ml). The inhibitor was in the vessel before the addition of the tissue and *p-cyclo*hexyloxy-α-phenylethylamine ($4{\cdot}5 \times 10^{-5}$M) was tipped from the side arm of the manometric vessel and incubated with the tissue for the times stated. Concentration ratios refer to mg drug in mg moist weight tissue/mg in 1 ml fluid. Respiration rates are given as μmoles/g moist weight/hr, and two respiratory rates joined by an arrow refer to respiration during the first and last 30 min of a 60 min experimental period. Slices which were incubated with the drug for 30 min were in contact with the inhibitor (in the bath at 37°) for 30 min prior to adding *p-cyclo*hexyloxy-α-phenylethylamine.

DISCUSSION

Fig. 8A shows the concentration ratios established by CHP, CHPA, and IPP, both *in vitro* and *in vivo*, the latter results being calculated from the results of BRIERLEY and MCCOUBREY (1953). The graph shows the concentration ratios established *in vitro* after a 60 min incubation period, and *in vivo* 10 min after intraperitoneal injection of the drug. Obvious differences are at once apparent: *in vitro* the analgesic compound is accumulated by cerebral cortex to a much greater extent than the non-analgesic, whereas *in vivo* there is little difference between their accumulation. Fig. 8B shows that the difference in accumulation between the analgesic and the non-analgesic compound is seen in heart and diaphragm as well as in cerebral cortex. It thus appears that *in vitro*, in contrast to *in vivo*, the analgesic compound is accumulated by the tissue more readily than the non-analgesic substance. Further differences between

TABLE 5.—EFFECTS OF WASHING ON ACCUMULATED *p-cyclo*HEXYLOXY-α-PHENYLETHYLAMINE AND *p-iso*PROPYLOXY-α-PHENYLETHYLAMINE

Drug	Concentration (M)	Washed	mg drug/ g wet wt.	Drug washed out (%)	Respiratory rates		No. obs.
					Before washing	After washing	
N	4×10^{-5}	—	0·048	—	82	—	2
N	4×10^{-5}	+	0·0106	78	80	72	2
A	4×10^{-5}	—	0·203	—	82	—	3
A	4×10^{-5}	+	0·138	33	81	70	3
—	—	+	—	—	81	69	1
N	4×10^{-4}	—	0·322	—	78	—	2
A	6×10^{-5}	—	0·220	—	78	—	2
N	4×10^{-4}	+	0·0947	68	72	54	2
A	6×10^{-5}	+	0·124	35	78	69	2
N	2×10^{-4}	—	0·248	—	76	—	1
A	6×10^{-5}	—	0·224	—	73	—	1
N	2×10^{-4}	+	0·057	77	74	66	1
A	6×10^{-5}	+	0·136	39	78	72	1

Slices of cerebral cortex (60 mg) from adult albino rats were incubated for 40 min in the absence of *p-cyclo*hexyloxy-α-phenylethylamine (A) or *p-iso*propyloxy-α-phenylethylamine (N). Some of the slices were then removed, washed in warm saline, placed in vessels containing warm drug-free medium and returned to the bath for a further 40 min. The incubation medium was Krebs–Ringer saline buffered with phosphate buffer, and the atmosphere in the vessels was O_2. In the table respiratory rates are given as μmoles/g moist wt./hr.

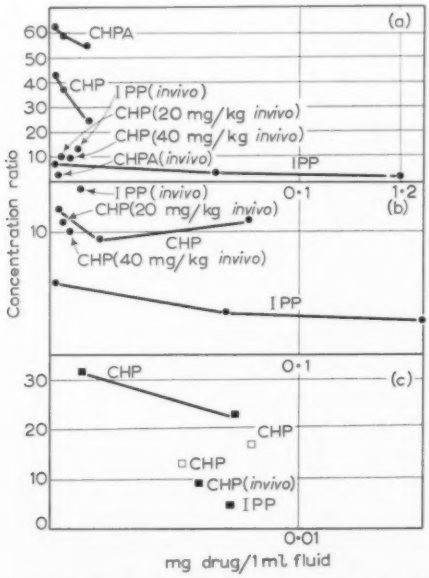

FIG. 8.—Accumulation of CHP, IPP and CHPA *in vivo* and *in vitro* by cerebral cortex slices (●), heart slices (□), or diaphragm (■). Results given are *in vitro* values except where otherwise stated. Graph's A and C give the *in vitro* results after 60 min incubation, and Graph B after 10 min. Conditions as in Fig. 5.

in vitro and *in vivo* uptake of the drugs are shown in Fig. 8A, B where it can be seen that the analgesic compound is accumulated to a greater extent *in vitro* than *in vivo*, while with the non-analgesic substance the reverse applies. These experiments show that the assumption that the ratio of drug between organ and plasma *in vivo* is equal to the ratio of drug between slice and saline *in vitro* does not necessarily hold true.

In Fig. 8C, the concentration ratios observed *in vitro* after a 10 min incubation period are plotted since, in the *in vivo* experiments, the tissues were removed 10 min after injection of the drug. In this case the values observed for the analgesic agent *in vitro* are very close to those observed *in vivo*, although the accumulation of IPP is still smaller *in vitro* than *in vivo*.

The reason for the difference between accumulation of CHP and IPP *in vitro* but not *in vivo* is not clear. It might be that *in vivo* and not *in vitro* the two compounds undergo metabolism to form the same or very similar substances. *In vivo* the maximum concentrating powers of the tissues will not be exerted since the drug is being continually excreted by the kidney and washed from the tissues by the circulating plasma. It may be, therefore, that as the maximum concentrating powers of the tissue have not been exerted the difference observed *in vitro* does not become apparent.

It is known that alteration in the size and shape of molecules can result in an altered accumulation of them by tissues. This has been noted in the present work; thus CHB, although fairly similar in structure to CHP, is concentrated by the tissue in greatly reduced amounts. The difference in accumulation of CHP and IPP *in vitro* could be accounted for by the slight difference in molecular structure. Further, it was found that the non-analgesic compound is more easily washed from the tissue slice than the analgesic compound. It appears reasonable to postulate, therefore, that the former compound is not so tightly bound in the tissue as the latter, which could account for its smaller accumulation *in vitro*.

Whatever is the mode of action of analgesic drugs it is necessary for the analgesic to reach its site of action. With the present compounds, CHP and IPP, it seems possible that while the latter compound is accumulated by the tissue it is not firmly bound to postulated 'receptors' in the cell and thus fails to exert an analgesic action. Experimental results obtained indicate that an energy source is not necessary for the accumulation of CHP. Thus, anaerobic conditions in the absence of substrate reduce the concentration ratio only partly. This small decrease could be due to alteration in the chemical constituents of the tissue cell, one of these constituents acting as a 'receptor' to the drug. MILLER and ELLIOTT (1954) likewise found that the accumulation of methadone by cerebral cortex slices was unaffected when glucose was omitted from the incubation medium and the experiment carried out under nitrogen.

The presence of inhibitors had no affect on accumulation of the drug. The small differences observed with substrates other than glucose can be accounted for by the greater alkalinity developing when these substrates are used, it having been shown that increased alkalinity increases the uptake of these drugs. It thus appears likely that the analgesic is accumulated by the slice by combining with some substance inside the cell, the concentration of 'free drug' inside the cell being equal to the concentration of 'free drug' outside the cell. The drugs are basic and combination with some acidic substance would be possible. The high concentration ratios found in suspensions of cerebral cortex point to the drug being combined with an insoluble component of the tissue such as nucleic acid, nucleoprotein or protein. There is

evidence that many drugs do combine with protein, for example sodium salicylate (SCAGLIONI, 1937), digitoxin (FAWAZ and FORAL, 1944), sulphanilamide (DAVIS, 1942), and some barbituric acid derivatives (BRODIE, 1954).

Previous determinations of the ability of analgesics to inhibit tissue metabolism have shown that they inhibit carbohydrate metabolism *in vitro* only at concentrations far in excess of those active *in vivo*. In most cases, however, accurate determinations of tissue drug concentrations have not been made, for example see QUASTEL and WHEATLEY (1932), SEEVERS and SHIDEMAN (1941, 1942), GREIG and HOWELL (1948) and WATTS (1949).

Considering first the metabolic effects of the drugs used in the absence of electrical pulses, it was shown that concentrations in the medium of CHP and IPP, which inhibit cerebral cortex slice respiration to a similar extent, were 4×10^{-4}M and $6{\cdot}15 \times 10^{-3}$M respectively. From these results it would appear that the analgesic is over ten times as effective in depressing respiration as the non-analgesic. Later experiments showed (Fig. 5) that the analgesic is concentrated by the slice to a much greater degree than the non-analgesic and, therefore, the former compound is only about twice as active as the latter in depressing respiration. These experiments emphasize the necessity for concentration studies before comparing metabolic effects of drugs.

It can be calculated from the results of BRIERLEY and MCCOUBREY (1953), that the concentration of CHP reached in cerebral cortex during analgesic action *in vivo* is approximately 0·18 μmoles/g moist weight. Taking the 10 min incubation period *in vitro*, which was shown earlier to be the most comparable to *in vivo* experiments Fig. 5 shows that the above concentration is reached in cerebral cortex slices when a concentration of drug between 2×10^{-5}M and 4×10^{-5}M is added to the slice. Fig. 3 shows that these concentrations do not affect the respiration of the slice.

Electrically stimulated tissue was found to be slightly more sensitive to the actions of the drugs used than tissue in the absence of pulses. CHP (10^{-4}M) practically completely depressed metabolic response to applied pulses and the concentration of drug in the slice, after a 10 min incubation period, was then 0·9 μmoles/g wet weight, which is not very far removed from the concentration of 0·18 μmoles/g wet weight found during analgesic action *in vivo*. CHP (6×10^{-5}M) partially inhibits the response to pulses and the concentration of drug in the slice would be in this case even nearer the *in vivo* concentrations.

Morphine (10^{-4}M) partially inhibits the response of cerebral cortex slices to stimulation, whilst 10^{-3}M drug practically abolishes it. MILLER and ELLIOTT (1955) state that injection of morphine (5 mg/kg) in cats produces complete analgesia between 30 and 120 min after injection and recovery is well under way at 150 min. It is probably more pertinent, therefore, to take the 30 or 60 min concentration ratios *in vitro* for comparison with the *in vivo* values. With morphine (10^{-4}M) a concentration of 0·24 μmoles/g wet weight is reached in the slice after a 30 min incubation period and 0·29 μmoles after 60 min. MILLER and ELLIOTT (1955) showed that the concentration of morphine in the cerebral cortex during analgesia *in vivo* is approximately 0·008 μmoles/g wet weight. Metabolic effects *in vitro*, therefore, are produced at higher concentrations than those producing analgesia *in vivo*. It is possible, however, that localization of the drug in specific areas of the tissue may occur *in vivo*. The concentrations of analgesics affecting metabolic events in the tissue *in vitro*

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are much closer to those active *in vivo* than has been found previously, even with separated enzyme systems.

It seems improbable that the analgesic mechanism is accounted for by the metabolic effects seen *in vitro* in the present experiments since although IPP was about half as effective in depressing tissue slice metabolism *in vitro* as CHP, much larger doses of the former than of the latter, *in vivo*, failed to produce analgesic effects (McCoubrey, 1953). It does appear likely, however, that some of the side effects seen with analgesics *in vivo*, for example narcotic and depressant effects, might be due to a depressant effect of the drugs on tissue respiration.

With respect to the depressant effects, it may be noted that CHP produces greater depression than IPP (McCoubrey, 1953) and this is consistent with the fact that the former compound has greater effect on the metabolic response to pulses than the latter.

SUMMARY

The accumulation of certain analgesics, non-analgesics and analgesic antagonists in cerebral cortex and some other tissues and their metabolic effects have been studied *in vitro*. Actions of the compounds on cerebral cortex and diencephalon were similar, while electrically stimulated tissue was more sensitive. The extent of the accumulation of drugs by tissues *in vitro* has been compared with accumulation *in vivo* and concentrations of drug effective *in vitro* approached those active *in vivo*. *p-cyclo*Hexyloxy-α-phenylethylamine, an analgesic, did not require an energy source for its accumulation and application of electrical pulses did not affect its uptake.

Acknowledgements—I am greatly indebted to Professor H. McIlwain for his advice and criticism during the course of this work, and to Dr. A. McCoubrey for the preparation of radioactive drugs. I also wish to thank the Medical Research Council for the grant of a scholarship held during these investigations, at The Institute of Psychiatry, Maudsley Hospital, Denmark Hill, London. The work reported here formed part of a thesis accepted by the University of London for the degree of Ph.D.

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Journal of Neurochemistry, 1958, Vol. 2, pp. 283–287. Pergamon Press Ltd., London

ACTIVATION AND OXIDATION OF LONG CHAIN FATTY ACIDS BY RAT BRAIN*

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(*Received* 23 *September* 1957)

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It is generally considered that the oxidation of fatty acids by brain tissue occurs to only a very limited extent or not at all (QUASTEL and WHEATLEY, 1933; WEINHOUSE, MILLINGTON and VOLK, 1950). It has been shown, however, that fatty acid oxidation can take place in brain slices and minces (GEYER, MATTHEWS and STARE, 1949; VOLK *et al.*, 1952). We have been interested in the metabolism of lipids by brain, and have studied the presence and distribution of some of the enzymes involved in the metabolism of long chain fatty acids. The extent of oxidation of long chain fatty acids by brain mitochondria has been followed with radioactive palmitic acid and palmityl coenzyme A. The results obtained are presented below.

MATERIALS AND METHODS

Tissue preparations. Unless otherwise stated, 15 to 20-day-old Sprague–Dawley rats were used in these experiments. Ten or 25 per cent rat brain homogenates were prepared in 0·25 M-sucrose and 0·001 M-versene, using a Potter–Elvehjem glass homogenizer, and cell fractions were obtained by differential centrifugation according to BRODY and BAIN (1952). Mitochondrial and microsomal fractions were washed once with half volumes of sucrose–versene and finally resuspended in the same volume of this medium, as was used for the original homogenate.

Materials. Coenzymes and fatty acids were obtained from commercial sources, and the latter were purified by distillation or recrystallization before use. Palmityl coenzyme A and palmityl adenylate were prepared according to VIGNAIS and ZABIN (1958).

Estimations. Fatty acid-activating enzyme was assayed with hydroxylamine by the method of KORNBERG and PRICER (1953). Palmityl coenzyme A dehydrogenase was measured with indophenol (GREEN *et al.*, 1954), using an acetone powder extract of brain mitochondria. The protein content of this extract was determined by a turbidimetric method similar to that of STADTMAN, NOVELLI and LIPMANN (1951). Fatty acid oxidation experiments were carried out with a Warburg apparatus. Respiratory carbon dioxide was collected, using filter paper impregnated with potassium hydroxide in the centre well. The reaction was stopped, and dissolved carbon dioxide was released from the medium and collected in the alkali by adding sulphuric acid from the side arm. Radioactivity in the respiratory carbon dioxide was determined by the elution of potassium carbonate from the paper, addition of carrier, and conversion to barium carbonate, which was plated and counted. Corrections were carried out by standard methods (REID, 1946).

The amount of radioactivity in water-soluble materials after incubation of labelled substrates was determined as follows: after removal of dissolved carbon dioxide, the incubation mixture was centrifuged, and the residue was washed with a small amount of water and centrifuged again. The combined supernatants were made strongly alkaline with concentrated ammonia, and the solution was shaken occasionally over a 30 min. period in order to hydrolyse palmityl coenzyme A. A small precipitate was removed by centrifugation. The clear solution was acidified with sulphuric acid, and was extracted three times with ethyl ether. An aliquot of the aqueous solution was dried on a planchet and counted. Controls were carried out by adding acid to the main compartment before incubation. Control values were usually very low or negligible.

* Supported by a grant, No. B 843 from the National Institutes of Health, U.S. Public Health Service.
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RESULTS

Distribution of palmitic acid-activating enzyme. Whole brain homogenates did not appear to catalyse the formation of palmityl hydroxamic acid when incubated with palmitate and other additions, as shown in Table 1. Separation of the homogenate into mitochondrial, microsomal and supernatant fractions, however, revealed that

TABLE 1.—ACTIVATION OF PALMITATE BY RAT BRAIN PREPARATIONS

Cell fraction	Palmityl hydroxamate formed (μM)
Homogenate	0
Mitochondria	0·33
Microsomes	0·29
Supernatant	1·12

1·0 ml of the cell fraction prepared from a 20 per cent homogenate in 0·25 M-sucrose and 0·001 M ethylenediaminetetra-acetate (EDTA) was incubated at 37° for 1 hr with 10 μM-crystalline adenosine triphosphate, 2,000 μM-hydroxylamine hydrochloride, pH 7·4, 100 μM-sodium fluoride, 30 μM-magnesium sulphate, 0·72 μM-coenzyme A, and 8 μM-potassium palmitate in a total volume of 4 ml. A blank determination was carried out for each fraction without fatty acid and coenzyme A. The values shown are net values and are the means of duplicate determinations.

the activating enzyme for palmitate was in fact present in brain, and was localized primarily in the supernatant fraction. The mitochondria and microsomes showed less activity.

Specificity of fatty acid-activating enzyme. The supernatant fraction of rat brain was incubated with a number of different fatty acids, and the formation of hydroxamic acids was measured (Table 2). It can be seen that of those fatty acids tested, the highest activity was found for stearic and palmitic acids.

TABLE 2.—SPECIFICITY OF FATTY ACID-ACTIVATING ENZYME OF RAT BRAIN SUPERNATANT

Experiment	Fatty acid	Hydroxamic acid formed (μM)
(1) 30 min	Laurate	0·22
	Myristate	0·66
	Palmitate	1·10
	Stearate	1·80
	Oleate	0·75
(2) 40 min	Butyrate	0·78
	Octanoate	0·61
	Laurate	0·24
	Palmitate	1·77

The enzyme was obtained by centrifuging a 25 per cent homogenate of rat brain in 0·25 M-sucrose and 0·001 M-EDTA for 30 min at 23,000 G. The supernatant (1·0 ml) was incubated at 37° with 8 μmoles of the indicated fatty acid and other additions as in Table 1. A blank determination was carried out without fatty acid and coenzyme A. The values shown are the means of duplicate determinations and are net values.

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Palmityl coenzyme A dehydrogenase. The presence of an enzyme which reduced indophenol with activated palmitate was easily demonstrated in extracts of acetone powder of brain mitochondria. The assay (GREEN *et al.*, 1954) was not carried out with palmityl coenzyme A as substrate, because the acetone powder extracts contained a hydrolase for this compound. Free coenzyme A, thus released, causes a non-enzymatic reduction of indophenol. Instead, palmityl adenylate was used as substrate, and

TABLE 3.—OXIDATION OF PALMITATE AND PALMITYL COENZYME A BY BRAIN MITOCHONDRIA

Age of rats	Tissue	Dry wt. (mg)	Total counts/min in CO_2		Total counts/min in water-soluble materials		Total counts/min	
			Palm.	Palm. CoA	Palm.	Palm. CoA	Palm.	Palm. CoA
18 days	Brain	47·7	920	490	740	1,190	1,660	1,680
15 days		46·8	275	185	335	815	610	1,000
8 months		49·5	805	430	325	835	1,130	1,265
8 months, starved		49·2	795	475	130	410	925	885
18 days	Liver	50·6	4,150	3,020	2,900	2,875	7,050	5,895

Incubations were carried out in Warburg vessels, each of which contained in the main compartment 0·5 ml mitochondria in 0·9 % KCl, 0·6 ml of 0·9 % KCl, 40 μM-phosphate buffer, pH 7·5, 5 μM-ATP, 6 μM-$MgCl_2$, 0·05 μM-cytochrome c, 120 μM-nicotinamide, 1 μM-DPN, 5 μM-glutathione, 0·5 μM-coenzyme A, 20-μM-malate, and 0·5 μM-[1–^{14}C] palmityl coenzyme A or [1–^{14}C] palmitate containing 56, 400 counts/min in a volume of 2·8 ml. The center well contained 0·2 ml 2N–KOH, and the side arm 0·4 ml N-H_2SO_4. Brain samples were incubated 70 min in air at 37° and the liver samples were incubated for 60 min before tipping in acid from the side arm. The values shown for radioactivity are net values obtained by deducting the counts obtained from controls which were carried out by adding acid to the main compartment before incubation.

a small quantity of coenzyme A was added. The 'residue fraction' of guinea pig liver of KORNBERG and PRICER (1953), which was found to catalyse a transfer of palmityl from adenylate to coenzyme A (VIGNAIS and ZABIN, 1958), was added in such quantity that the formation of palmityl coenzyme A could not be rate-limiting. The extract was obtained by stirring 15 mg of acetone powder of brain mitochondria for 1 hr at 0° with 1·0 ml of 0·1 M-2-amino-2-hydroxymethylpropane-1:3-diol (Tris) buffer, pH 7·0. To 0·3 ml of the clear solution obtained by centrifugation were added 20 μM-Tris buffer, pH 7·0, 0·5 μM-palmityl adenylate, 0·04 μM-coenzyme A, 1 mg of residue fraction of guinea pig liver, and 0·6 ml of 0·01 % 2:6-dichlorophenol indophenol in a final volume of 2·8 ml. The rate of reduction of the dye was followed against two controls, one of which omitted palmityl adenylate, and the other which omitted brain extract. The change in optical density per mg of brain mitochondria protein at 600 mμ was 0·075/min. This activity is actually about 50 per cent greater than that obtained by GREEN *et al.* (1954) for an acetone powder extract of beef liver particles containing butyryl coenzyme A dehydrogenase.

Oxidation of palmitate by brain. Several attempts to measure oxidation of fatty acids by brain mitochondria using manometric techniques were carried out, but although the data occasionally indicated that oxidation had occurred, no clear-cut

evidence was obtained that this was true. The more sensitive tracer method was therefore used. Using carboxyl-labelled palmitate and palmityl coenzyme A it was shown by measurement of radioactivity in the respiratory CO_2 that oxidation of these substrates had occurred (Table 3). Further, some radioactivity from the fatty compounds was found after incubation to be in water-soluble materials not extracted by ether, and these must have included some intermediates in the oxidation of palmitate.

Both labelled palmitate and its coenzyme A derivative were tested because the fatty acid-activating enzyme was found to be relatively low in activity in mitochondria. Unexpectedly, the conversion of palmitate to carbon dioxide was greater than the conversion of palmityl coenzyme A to carbon dioxide. The reverse was true, however, for the water-soluble substances, so that the sum of radioactivity in the fractions isolated was approximately the same, whether the substrate was palmitate or palmityl coenzyme A. No marked differences in oxidation were observed when mitochondria from the brain of older rats and of older, starved rats was used.

An experiment carried out under the same conditions but using liver mitochondria is also shown in Table 3. The amount of isotope found in respiratory carbon dioxide and the water-soluble materials was about five or six times higher than with the brain preparation.

DISCUSSION

The existence in brain of certain enzymes active in the metabolism of lipids suggests that the brain may not be as inert towards these materials as generally believed. The data reported here have shown a relatively strong activating enzyme for long chain fatty acids, which is similar to the enzyme discovered by KORNBERG and PRICER (1953) in guinea pig liver. It is interesting to note that the optimum activity for the brain enzyme occurs for longer chain fatty acids than is true for the liver enzyme, which has optimum activity for lauric acid. This is interesting in view of the fact that brain tissue contains very long chain fatty acids which are present in lower concentrations in liver. No trace of the activating enzyme could be found in the complete homogenate, yet when this was separated by centrifugation, activity was present in both supernatant and particulate fractions. This observation can probably be explained by the suggestion that additional enzymes, which can catalyse acyl transfer, are also present in brain. In a complete system, activation and acyl transfer to other, natural acceptors may occur more readily than to hydroxylamine, while in the separated fractions the only reaction that can go on is the activating reaction and subsequent hydroxamate formation.

By direct assay, an enzyme which reduced indophenol in the presence of palmityl coenzyme A was shown to occur in acetone powder extracts of brain mitochondria. The product of this reaction must have been Δ^2-hexadecenoyl coenzyme A by analogy with the same step in fatty acid oxidation, which has been studied in greater detail with shorter chain fatty acids. Indirect assay for the other enzymes of the fatty acid spiral, carried out by measuring radioactive carbon dioxide and water-soluble materials formed from labelled palmitate or palmityl coenzyme A, indicates that these are also present in brain tissue.

As expected, the oxidation of these substrates by liver proceeded five or six times faster than the oxidation by brain. It should be noted that the conditions used were not necessarily optimal for either tissue. For example, low levels of substrate were added in order to conserve material. It is clear, however, that the oxidation of fatty acids in brain is far less than in other tissues, and it is probable that one or more of the

enzymes which act later in the oxidative sequence is present in limiting amounts. LYNEN (1954), using short chain compounds as substrates, has shown that β-keto-reductase and β-ketothiolase are present in very low concentrations in brain.

Radioactive palmityl coenzyme A also was used in the oxidation experiments because the amount of activating enzyme found in brain mitochondria was fairly low. The data showed that the conversion of palmitate to carbon dioxide was greater than the conversion of palmityl coenzyme A to carbon dioxide. On the other hand, more water-soluble products were obtained from palmityl coenzyme A than from palmitate. It is difficult to explain these observations, but it is possible that the preparation of radioactive palmityl coenzyme A used here contained an inhibitor. Another explanation may be that oxidative products of palmityl coenzyme A metabolism, which would reach a higher concentration than could be obtained from palmitate, might inhibit certain of the final steps in the oxidation sequence. Although the existence of certain enzymes in brain offers an enzymatic mechanism for reaction sequences, it should be emphasized that this does not prove that these reactions also occur *in vivo*.

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SUMMARY

(1) An enzyme that activates long chain fatty acids has been shown to be present in rat brain: it is found in highest quantity in the supernatant fraction obtained by the centrifugation of sucrose homogenates.

(2) Palmityl coenzyme A dehydrogenase activity is present in rat brain mitochondria.

(3) Radioactive palmitate and palmityl coenzyme A were shown to be converted to radioactive carbon dioxide and water-soluble materials by brain mitochondria. The rate of these conversions with brain tissue was one-fifth to one-sixth of that with liver.

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ERRATUM

LAJTHA A., FURST S., GERSTEIN A. and WAELSCH H. (1957) *J. Neurochem.* **1,** 289. *Amino Acid and Protein Metabolism of the Brain—I.*

On p. 297, Table 6:

In the heading of Table 6, in place of (t) it should read ($t_{\frac{1}{2}}$).

In the footnote to Table 6, instead of $F =$ γ lysine/mg protein/min., it should read $F =$ γ lysine/g protein/min.

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Journal of Neurochemistry, 1958, Vol. 2, pp. 289 to 294. Pergamon Press Ltd., London

LIPIDS IN THE BRAINS OF INFANTS AND CHILDREN

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(*Received* 6 *December* 1957)

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ONE of us (CUMINGS, 1953) has published the results of estimations of various lipid substances found in the normal adult human brain as well as in brains from patients with various diseases. Some of the latter brains were obtained from children but there are few comprehensive studies of the normal young developing brain.

JOHNSON, MCNABB and ROSSITER (1948) examined a few brains of stillborn infants, and they compared their results with those obtained in adult material. SCHUWIRTH (1940) and BRANTE (1949) have also studied a few such cases, but their results are not in complete agreement. Accordingly, over the past few years we have made a study of some of the cerebral lipids in infants and young children, and we are presenting the results in this paper.

MATERIALS AND METHODS

Brains were obtained at autopsy from the foetus, from prematurely born infants, from infants dying at or just before birth, and from children of up to 12 years of age. The cerebral cortex and the cerebral white matter were very carefully separated and the lipids estimated on each portion. Two foetal brains, one of 10 weeks, and the other of 28 weeks could not be examined in this manner, and in these the lipids of the whole brain were estimated. All the brains examined were in the unfixed state and formalin-fixed tissues were not used in this study.

The lipid was extracted, and the water content, total phospholipid, sphingomyelin, lecithin, kephalin, total and free cholesterol, and 'galactose cerebrosides' were estimated as already described (CUMINGS, 1953). Neuraminic acid (sialic acid) and total hexosamine were determined by the methods given by CUMINGS (1957). The true cerebroside content was obtained using the technique of RADIN, LAVIN and BROWN (1955).

RESULTS

Tables 1 and 2 give the figures obtained for the phospholipids, cholesterol and water content of the cerebral white matter, and the cerebral cortex respectively.

It can be seen that there is a steady increase in the phospholipid components with increasing age with, at the same time, a decrease in the water content of the brain, and that at around the age of 12 years the results are comparable with those found in the adult. Total cholesterol also increases in a like manner, and at no age is there evidence of any appreciable amount of esterified cholesterol (See Figs. 1 and 2). Contrary to the findings of SCHUWIRTH (1940) sphingomyelin was found to be present in both the foetus and the neonate; these findings agree with those of BRANTE (1949).

EDGAR (1956) has criticized the use of the term 'cerebrosides' for the substances which both JOHNSON *et al.* (1948) and one of us have estimated previously, for he has pointed out that substances other than true cerebrosides are estimated by the method that was used. We are aware of this defect, and are using the term 'galactose cerebrosides' to indicate that they have been calculated from the galactose equivalents.

TABLE 1.—LIPID CONTENT OF CEREBRAL WHITE MATTER
(g/100 g fresh tissue)

Age and maturity	Total phospho-lipid	Sphingo-myelin	Kephalin	Lecithin	Cholesterol		Water
					Total	Esterified	
10 weeks foetus*	1·43	0·32			0·18	0	91·5
28 weeks foetus*	1·07	0·5	0·28	0·34	0·45	0·03	93·7
34 weeks foetus	2·1	0·2	0·9	1·0	0·65	0	89·7
Full term (38 weeks)	2·1	0·4	1·0	0·7	0·5	0	91·0
Full term (5 cases)	1·95–2·5	0·2–0·8	0·5–1·3	0·7–1·1	0·65–0·87	0·01–0·15	75·8–91·0
1 day	2·0	0·1	1·2	0·7	0·74	0·04	89·0
4 days	3·2	0·30	1·4	1·5	1·15	0	91·8
7 days	2·8	0·5	1·3	0·9	0·55	0	87·7
2 months	2·8	0·3	1·7	0·8	0·9	0·1	89·0
3 months	3·7	1·0	1·6	1·1	1·15	0	86·8
4 months	4·5	0·7	2·5	1·3	1·7	0	82·3
7 months	3·2	0·8	1·1	1·3	1·8	0	81·4
10 months	4·8	1·1	2·5	1·2	2·4	0·1	79·8
2 years	4·9	1·1	2·5	1·3	3·2	0·1	75·7
3 years	3·0	0·7	1·5	0·8	2·1	0	74·9
5 years	4·7	1·2	2·8	0·7	3·7	0·15	71·2
6 years	6·0	1·4	3·2	1·4	4·4	0·05	73·0
7 years	6·6	1·6	3·4	1·6	4·5	0	71·3
12 years	6·3	2·0	2·8	0·5	3·8	0	74·8

*Whole brain.

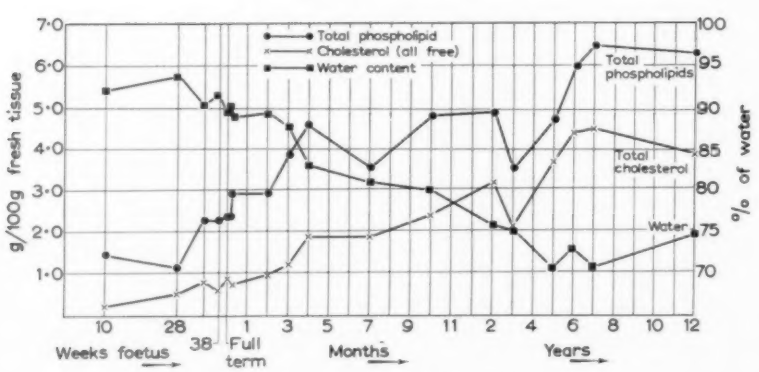

FIG. 1. Lipids in cerebral white matter.

TABLE 2.—LIPID CONTENT OF CEREBRAL CORTEX
(g/100 g fresh tissue)

Age and maturity	Total phospho-lipid	Sphingo-myelin	Kephalin	Lecithin	Cholesterol		Water
					Total	Esterified	
34 weeks foetus	1·75	0·25	0·7	0·8	0·5	0	91·5
Full term (38 weeks)	1·8	0·5	0·7	0·6	0·5	0	89·4
Full term (5 cases)	1·8–2·5	0·3–0·4	0·8–1·8	0·2–1·0	0·54–0·67	0–0·02	85·5–89·2
1 day	1·8	0·1	1·4	0·3	0·53	0	88·9
4 days	3·0	0·3	1·3	1·4	1·0	0	91·4
7 days	2·5	0·5	1·2	0·8	0·47	0·02	90·4
2 months	2·7	0·3	1·5	0·9	0·7	0	90·0
3 months	2·7	0·4	1·3	1·0	0·6	0·01	88·5
4 months	2·9	0·3	1·6	1·0	0·7	0	83·3
7 months	2·3	0·6	0·8	0·9	0·8	0	87·0
10 months	2·5	0·5	1·3	0·7	0·8	0	88·5
2 years	2·3	0·5	1·1	0·7	0·9	0	84·7
3 years	2·7	0·5	1·3	0·9	1·1	0	84·1
5 years	2·7	0·7	1·5	0·5	1·0	0	84·9
6 years	3·5	0·6	1·7	1·2	1·0	0	84·2
7 years	3·2	0·7	1·7	0·8	0·9	0	83·7
12 years	4·0	0·8	2·2	1·0	0·9	0·06	78·9

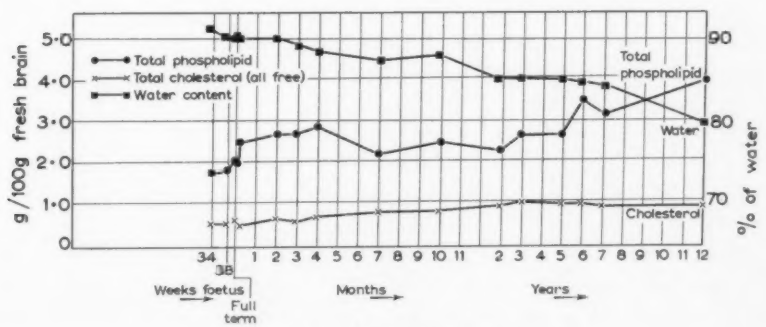

FIG. 2. Lipids in cerebral cortex.

Accordingly, a series of brains from these same infants have been examined both for their 'galactose cerebroside' content and for the true cerebroside level (RADIN *et al.*, 1955) and Table 3 shows the results obtained.

The feature of chief interest in Table 3 is that at all ages there is a marked difference in the two sets of figures for the cerebral cortex. This is probably due to the presence of hexoses in the gangliosides of the cortex, but this will be discussed more fully in a later paper. The figures obtained in the cerebral white matter demonstrate that, as the age increases, there is much less discrepancy between the two sets of results.

TABLE 3.—CEREBROSIDES IN BRAIN TISSUE
(g/100 g fresh tissue)

Age and maturity	Cerebral white matter		Cerebral cortex	
	'Galactose cerebroside'	Cerebroside (RADIN *et al.*)	'Galactose cerebroside'	Cerebroside (RADIN *et al.*)
Full term (1)	0·54	0·05	0·46	0·04
(2)	0·39	0·03	0·34	0·04
(3)	0·70	0·05	0·5	0·07
(4)	0·7	0·04	0·6	0·03
(5)	0·6	0·09	0·5	0·07
1 day	0·5	0·09	0·6	0·06
4 days	0·48	0·08	0·72	0·06
7 days	0·32	0·07	0·37	0·11
2 months	1·1	0·4	0·9	0·2
3 months	1·15	0·53	0·48	0·04
7 months	2·23	1·3	0·43	0·14
10 months	2·5	2·15	0·48	0·03
2 years	3·60	1·9	0·70	0·14
3 years	3·13	1·23	1·03	0·29
5 years	4·7	3·17	0·61	0·07
6 years	4·6	4·0	0·79	0·2
7 years	5·15	5·02	0·67	0·19
12 years	4·03	3·31	0·91	0·26

There is very little difference in cerebroside content between cortex and white matter until the infant is a few months old, and this is also true of the sphingomyelin content.

The results of neuraminic acid (sialic acid, see BLIX, GOTTSCHALK and KLENK, 1957) and of hexosamine estimations in the cerebral cortex and in the white matter respectively are given in Table 4.

It is of interest to note that the hexosamine level is highest from just before birth up to 7 months, while the neuraminic acid level is highest between 3 months and 7 years (See Fig. 3).

DISCUSSION

The pattern of distribution of phospholipids, of cholesterol and of cerebrosides given in this paper agrees with the findings of JOHNSON *et al.* (1948) and of BRANTE (1949), although a far wider range of ages has been covered. It is of considerable interest that sphingomyelin and true cerebroside in the white matter increase in amount at a rate corresponding to the degree of myelination shown histologically. This would be expected since these two substances make up a large part of the total solid matter of the myelin sheath, and equally it is not surprising that there is relatively less difference in the amounts of these two substances at different ages in the cerebral cortex.

Now that one has a series of normal controls, obtained the two methods for estimating cerebroside, it will be of considerable interest to make comparable examinations in the various pathological conditions in which one might expect to find, or in which there has already been found, a raised cerebroside level. Such examples

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TABLE 4.—TOTAL HEXOSAMINE AND NEURAMINIC ACID IN BRAIN TISSUE (mg/100 g dry tissue)

Age and maturity	Cerebral white matter Total hexosamine	Cerebral cortex Neuraminic acid
10 weeks foetus*	410	109
28 weeks foetus	300	208
34 weeks foetus	647	200
38 weeks foetus	622	223
Full term (5 cases)	335–556	217–319
4 days	573	162
7 days	750	270
2 months	612	328
3 months	705	323
4 months	608	347
7 months	410	478
10 months	254	355
2 years	278	377
3 years	232	396
5 years	227	413
6 years	217	351
7 years	239	552
12 years	284	223

* Whole brain.

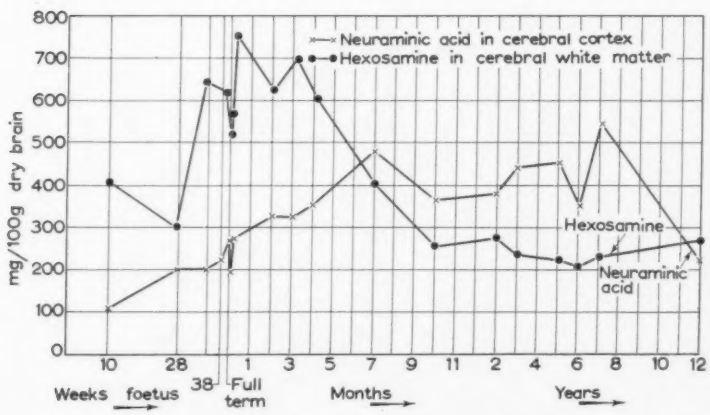

FIG. 3. Neuraminic acid and hexosamine in brain.

can be found in Niemann Pick's disease and in Gaucher's disease when the brain is affected.

No previous figures were available for hexosamine and gangliosides in a series of children of various ages, and it is of interest to note that the peaks of the increase occurred at different ages. EDGAR (1956) has suggested that the estimation of hexosamine would be a better guide to the ganglioside level than estimations of neuraminic acid, but this is obviously not correct in view of our findings, even though it might be true to say that the difference between 'galactose cerebrosides' and true cerebrosides in

the cerebral cortex may be accounted for by the hexose content of ganglioside. However, this may not necessarily be true as is seen if one compares even superficially the figures presented in Tables 3 and 4. It would appear unlikely that the hexosamine figures in the white matter bear any absolute relationship to either the cerebrosides or to the gangliosides. These results have been found to be of value in the examination of cerebral biopsy material (CUMINGS, 1957).

SUMMARY

Cerebral lipids, including hexosamine and neuraminic acid have been estimated in human brains from a foetal age of 10 weeks to a child of 12 years.

Sphingomyelin and cerebroside have been shown to increase in amount in the white matter at a rate expected for components of myelin.

Hexosamine and neuraminic acid increase in amount in the white matter and in the cortex respectively and a peak is reached in each at a different period of growth.

Acknowledgements—Our thanks are due to the many pathologists who have assisted us in collecting suitable material. We also wish to thank the Research Advisory Committee for their help.

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CHANGES IN THE AMMONIA AND GLUTAMINE CONTENT OF THE RAT BRAIN INDUCED BY ELECTRIC SHOCK

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(*Received* 30 *December* 1957)

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IT has been known for some time that ammonia is formed explosively in brain tissue post mortem, and that the formation of ammonia can also be observed *in vitro* with respiring brain slices in the absence of substrate (RICHTER and DAWSON, 1948; WEIL-MALHERBE and GREEN, 1955; TSUKADA and TAKAGAKI, 1954; TAKAGAKI, HIRANO and TSUKADA, 1957). These findings are peculiar to brain tissue and are not shown by other tissues. Ammonium salts may be of special importance in view of their activity as cerebral irritants, and glutamine may participate in the detoxication of ammonia in the brain (ELLIOTT, 1951; WEIL-MALHERBE, 1950).

The normal ammonia content of the rat brain has been established by RICHTER and DAWSON (1948) using the rapid freezing technique. The ammonia and glutamine content of the living rat brain can now be estimated accurately in various states of cerebral activity, and the ammonia level in the brain has been regarded as a biochemical indicator giving an accurate index of the state of physiological activity of the brain (VLADIMIROV, 1953; BENITEZ, PSCHEIDT and STONE, 1954).

The present paper gives the results of experiments on the effect of electrical stimulation, applied under various conditions, on the brain ammonia and glutamine levels. A preliminary account of these experiments has been published (TSUKADA, TAKAGAKI, HIRANO and SUGIMOTO, 1956).

METHODS

Experimental arrangements. For the stimulation and fixation of the animals the method of VLADIMIROVA (1954) was followed with the aid of the apparatus shown in Fig. 1. The apparatus was set up in a dark and quiet room. When the experimental animal was put in the chamber *a*, it showed the orientation reflex for 30–60 min. After the animal had become calm, the electric magnet *d* was switched off and the animal dropped into liquid air. In this way the ammonia and glutamine concentrations in the brain of resting animals protected from all outside stimuli were obtained.

Animals taken after the disappearance of the orientation reflex were stimulated by an electric current (30–50 v a.c.) applied for various periods to the paws through the grid *b*. By this method of stimulation through the skin receptors, the experimental animals showed an active defence reaction without convulsive seizures.

The experimental animals used were rats of both sexes of approximately 50–70 g in weight. They were kept for several days before the experiment, in a common cage and fed on an ordinary solid diet.

Chemical analyses. The brains of the rats frozen in liquid air were rapidly chiselled out, ground

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to a powder at a low temperature, transferred to a weighed flask containing 3 ml of 10% trichloroacetic acid, and homogenized. The wet weight of brain tissue was determined. After centrifugation, determinations were carried out on the supernatant solution.

The ammonia content of the sample was determined by the Conway microdiffusion technique using a diffusion time of 60 min at 37° and half-saturated K_2CO_3 (RICHTER and DAWSON, 1948). The amount of ammonia liberated by hydrolysis of the brain glutamine under these conditions was estimated and a correction was applied to the brain ammonia values on this basis. The glutamine content was determined separately by the method of HARRIS (1943), essentially as described by RICHTER and DAWSON (1948). The ammonia liberated on hydrolysis with 10% trichloroacetic acid at 70° for 75 min was determined unless otherwise stated. In some cases glutamine was determined by estimating the ammonia liberated on hydrolysis with half-saturated KOH at 37° for 60 min (MESSER, 1955).

RESULTS

1. *Effects of stimulation and narcosis on the concentrations of ammonia and glutamine in the rat brain.* The mean value of 0·36 μmole/g obtained for the ammonia content of rats in the resting state was significantly higher than the value reported by RICHTER and DAWSON (1948). This higher value may be attributed mainly to the higher body weight of the rats used, since it takes a longer time for the fixation of larger animals with liquid air. On the other hand the mean value of 3·15 μmole/g for glutamine was lower than that obtained by RICHTER and DAWSON (1948); but this lower level was a consistent finding in the present experiments (Table 1).

Electrical stimulation for 5 sec resulted in a significant increase of the ammonia concentration in the rat brain and a mean of 0·45 μmole/g was obtained. With stimulation of duration longer than 5 sec, this increase in ammonia level was not observed. Stimulation applied only for 1 sec induced a significant decrease in the ammonia concentration.

Rats anaesthetized for 30 min by subcutaneous injection of urethane or methylhexabarbital showed lower brain ammonia values, the mean levels being 0·27 or 0·23 μmole/g respectively.

TABLE 1.—AMMONIA AND GLUTAMINE CONTENT OF THE RAT BRAIN UNDER VARIOUS CONDITIONS

Condition	No.	Ammonia (μmole/g ± S.D.)	Glutamine (μmole/g ± S.D.)
Resting state	14	0·36 ± 0·07	3·15 ± 0·69
Elect. stim. for 1 sec	6	0·27 ± 0·06*	3·01 ± 0·28
5 sec	11	0·45 ± 0·01†	3·01 ± 0·46
15 sec	4	0·36 ± 0·09	2·83 ± 0·33
30 sec	4	0·39 ± 0·06	3·51 ± 0·89
60 sec	4	0·37 ± 0·01	3·40 ± 0·24
120 sec	5	0·38 ± 0·08	2·91 ± 0·49
Anaesthesia (urethane)	7	0·27 ± 0·03*	3·65 ± 1·27
(methylhexabarbital)	4	0·23 ± 0·04†	2·96 ± 0·39
Conditioned stimulus (light)	5	0·46 ± 0·09*	2·99 ± 0·88

Mean values are followed by standard deviations. The asterisks and daggers show significant differences of the experimental value from the control resting value (0·36 ± 0·07 μmole/g for brain ammonia).

* $P \leq 0{\cdot}02$,

† $P < 0{\cdot}01$.

The lighting of a 60 w electric lamp for 5 sec as a conditioned stimulus was associated with the electrical stimulation for 5 sec. For this purpose, the larger rats (about 70 g in weight) were used. The stimulus was reinforced ten times a day for 10 days (apparatus, see Fig. 1). After establishment of the conditioned reflex, the rats were stimulated only by the light for 5 sec and then they were dropped into liquid air. In these experiments a rise in brain ammonia was observed as in the case of 5 sec unconditioned electrical stimulation.

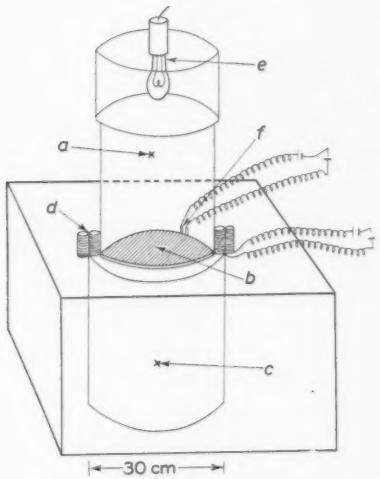

FIG. 1.—Experimental apparatus. *a:* glass cylinder; *b:* grid; *c:* vacuum flask (made of copper); *d:* electric magnet; *e:* electric lamp and lamp-house; *f:* stimulating circuit.

Under the above experimental conditions, significant changes in the glutamine level were not observed.

2. *After-effect of electrical stimulation and effect of a second stimulation.* The increased level of ammonia in the rat brain caused by a 5 sec electrical stimulation returned to the resting level within 60 sec after stimulation. At 15 sec after stimulation, however, the ammonia still showed a higher value than the normal, the mean being 0·43 μmole/g (Table 2).

A second electrical stimulation applied within 120 min after the first one of 5 sec duration did not induce an increase in the ammonia content. When a second electrical stimulation of 5 sec duration was given 15 sec after the first electrical stimulation, the increased level of ammonia returned to the normal resting figure. A second electrical stimulation of 5 sec duration increased the ammonia content as usual at 150 min after the first stimulation. From the data given above, it would appear that the first 5 sec electrical stimulation affects the chemical processes concerned in the ammonia metabolism of the brain for a long period without changing the ammonia content.

The glutamine concentration did not change significantly under these experimental conditions.

TABLE 2.—EFFECTS OF A SECOND ELECTRICAL STIMULATION ON THE AMMONIA AND GLUTAMINE CONTENT OF THE RAT BRAIN

Time after first elect. stim.	Second stim.	No.	Ammonia (μmole/g $\pm$ S.D.)	Glutamine (μmole/g $\pm$ S.D.)
15 sec	−	6	0·43 $\pm$ 0·09*	3·65 $\pm$ 0·57
	+	5	0·37 $\pm$ 0·04	3·55 $\pm$ 0·34
60 sec	−	4	0·32 $\pm$ 0·01	3·16 $\pm$ 0·47
	+	5	0·32 $\pm$ 0·10	3·56 $\pm$ 0·21
120 sec	−	4	0·34 $\pm$ 0·04	3·15 $\pm$ 0·47
	+	4	0·34 $\pm$ 0·03	2·93 $\pm$ 0·65
10 min	+	4	0·37 $\pm$ 0·02	3·19 $\pm$ 0·44
60 min	+	4	0·36 $\pm$ 0·04	4·05 $\pm$ 0·97
120 min	+	4	0·40 $\pm$ 0·14	4·53 $\pm$ 1·35
150 min	+	4	0·51 $\pm$ 0·05†	2·99 $\pm$ 0·28

Statistical procedure as in Table 1.
* $P = 0.05$,
† $P < 0.01$.
(Control resting value for brain ammonia, 0·36 $\pm$ 0·07 μmole/g.)

3. *The effects of ammonium chloride administration and of electrical stimulation.* Sixty minutes after the subcutaneous injection of 20 mg NH_4Cl per kg of body weight, there were found to be no significant changes in the brain ammonia and glutamine levels. But when electrical stimulation for 5 sec was applied 60 min after injecting NH_4Cl, an increase in the brain ammonia was not observed (Table 3). However, after subcutaneous injection of 0·2 ml distilled water instead of NH_4Cl, a marked increase in the brain ammonia was induced by the electrical stimulation for 5 sec. Therefore, the injection itself had no effect on the brain ammonia. No changes in glutamine concentration were found. Furthermore, even when a large dose of

TABLE 3.—EFFECTS OF NH_4Cl INJECTION ON AMMONIA AND GLUTAMINE CONTENT OF THE RAT BRAIN

Injection	Elect. stim.	No.	Ammonia (μmole/g $\pm$ S.D.)	Glutamine (μmole/g $\pm$ S.D.)
NH_4Cl, 20 mg/kg (subcutaneously)	−	6	0·32 $\pm$ 0·09	3·27 $\pm$ 0·24
	+	6	0·33 $\pm$ 0·06	3·34 $\pm$ 0·24
NH_4Cl, 200 mg/kg (intraperitoneally)	−	9	0·38 $\pm$ 0·10	5·63 $\pm$ 1·31*
H_2O, 0·2 ml	+	11	0·43 $\pm$ 0·08†	3·87 $\pm$ 0·51

Statistical procedure as in Table 1.
* $P < 0.01$,
† $0.02 < P < 0.05$.
(Control resting value, 0·36 $\pm$ 0·07 μmole/g for ammonia and 3·15 $\pm$ 0·69 μmole/g for glutamine.)

TABLE 4.—EFFECT OF CONTINUOUS ELECTRICAL STIMULATION FOR 30 min ON THE GLUTAMINE CONTENT OF THE RAT BRAIN

Condition	No.	Ammonia (μmole/g $\pm$ S.D.)	Glutamine* (μmole/g $\pm$ S.D.)
Resting state	11	0·34 $\pm$ 0·04	3·87 $\pm$ 0·39
Stimulated for 30 min	6	0·36 $\pm$ 0·05	4·37 $\pm$ 0·33†

* Values obtained by hydrolysis with half-saturated KOH.
† $P = 0{\cdot}02$

NH_4Cl (200 mg/kg) was administered to rats intraperitoneally, the brain ammonia did not change significantly; but the content of brain glutamine was increased significantly (mean value 5·63 μmole/g).

These experiments suggest that the suppression of a rise in ammonia content for a period of 120 min after a first electrical stimulation of 5 sec is related to the increase in ammonia concentration following to the first stimulation.

4. *Effect of continuous electrical stimulation on the ammonia and glutamine concentrations of the brain.* After the rats had been put into the apparatus, they were stimulated continuously through their paws for 30 min with 30 v (a.c.) The glutamine content of the brain increased significantly, while there was no significant change in the ammonia concentration (Table 4).

Accordingly, it may be thought that the extra ammonia liberated in the brain by the stimulation is fixed by glutamic acid and accumulated as glutamine.

5. *Effect of repeated electrical stimulation on the brain ammonia level.* Rats stimulated electrically ten times a day at random for 10 days reacted more rapidly with defence behaviour.

In rats conditioned as above, the second electrical stimulation of 5 sec duration evoked a significant rise in the brain ammonia 60 min after the first stimulation, but not 30 min after (Table 5). They also responded to electrical stimulation for 5 sec

TABLE 5.—EFFECT OF ELECTRICAL STIMULATION ON THE AMMONIA AND GLUTAMINE CONTENT OF THE BRAIN IN ELECTRICALLY CONDITIONED RATS

Condition	No.	Ammonia (μmole/g $\pm$ S.D.)	Glutamine* (μmole/g $\pm$ S.D.)
Second elect. stimulus 30 min after first	4	0·33 $\pm$ 0·04	3·64 $\pm$ 0·36
Second elect. stimulus 60 min after first	6	0·49 $\pm$ 0·04†	4·08 $\pm$ 0·37
Elect. stimulus 60 min after injection of NH_4Cl	6	0·41 $\pm$ 0·08‡	3·67 $\pm$ 0·29
Resting state	14	0·34 $\pm$ 0·06	3·43 $\pm$ 0·55

* Values determined by hydrolysis with KOH.
† $P < 0{\cdot}01$.
‡ $0{\cdot}02 < P < 0{\cdot}05$.

with an increase in brain ammonia 60 min after the injection of NH_4Cl (20 mg/kg). The time interval required for return after stimulation to the normal state of the brain appeared to be shortened in the repeatedly stimulated rats.

There were no changes in the glutamine concentration under these experimental conditions.

6. *Effects of repeated administration of* NH_4Cl. Rats conditioned by the subcutaneous injection of NH_4Cl (30 mg/kg) once a day for 10 days, showed no significant changes in the brain ammonia or glutamine 60 min after electrical stimulation for 5 sec (Table 6). However, an increase in brain ammonia by a second electrical stimulation was induced already at 60 min after the first one in rats conditioned with NH_4Cl,

TABLE 6.—EFFECT OF ELECTRICAL STIMULATION ON AMMONIA AND GLUTAMINE CONTENTS IN THE BRAIN OF RATS REPEATEDLY INJECTED WITH NH_4Cl

Condition	No.	Ammonia (μmole/g $\pm$ S.D.)	Glutamine* (μmole/g $\pm$ S.D.)
60 min after first elect. stimulus in repeatedly injected rats with NH_4Cl	6	0·34 $\pm$ 0·05	3·38 $\pm$ 0·28
Second elect. stimulus 60 min after first one in repeatedly injected rats with NH_4Cl	11	0·42 $\pm$ 0·10†	3·84 $\pm$ 0·14
Second elect. stimulus 60 min after first one in repeatedly injected rats with H_2O	7	0·38 $\pm$ 0·06	3·43 $\pm$ 0·33
Resting state‡	14	0·34 $\pm$ 0·06	3·43 $\pm$ 0·55

* Values determined by hydrolysis with KOH.
(Statistical procedure as in Table 5.)
† $P < 0·02$.
‡ Same as Table 5.

as it was in electrically conditioned animals (Table 6). On the other hand, rats repeatedly injected with distilled water (0·2 ml) once a day for 10 days, did not show a rise in brain ammonia even after a second electrical stimulation applied 60 min after the first one. The glutamine concentration did not change under these conditions.

Therefore, it was shown that the time course of the recovery processes in brain is hastened by repeated injection of NH_4Cl as well as by repeated electrical stimulation.

DISCUSSION

It is well known that ammonium ions play an important role in nervous tissues and that the concentration in the brain is closely related to the state of activity; that is, the ammonia level in the brain is increased in the excited state, and decreased in the anaesthetized state (RICHTER and DAWSON, 1948; TASHIRO, 1922; WEIL-MALHERBE, 1950; VLADIMIROV, 1953; STONE, 1955).

The source and mechanism of ammonia formation in brain tissue *in vivo* have not yet been clarified, although the formation of ammonia in respiring brain slices in the

absence of substrate has been regarded as due to the oxidative deamination of glutamic acid (TAKAGAKI, HIRANO and TSUKADA, 1957). VRBA (1955*b*, 1956) emphasized recently that the source of ammonia liberated during physical exertion by swimming might be the amide nitrogen of the protein fraction of the rat brain.

It has been shown that in order to obtain reliable values for the ammonia content of the living brain, the tissues of the experimental animals must be rapidly fixed by freezing in liquid air, so as to avoid post mortem changes (RICHTER and DAWSON, 1948; VLADIMIROVA, 1953). By the present procedures, it was thought to be possible to determine the ammonia level in the brain under various different physiological conditions, avoiding extrinsic influences as completely as possible. It was shown that the concentrations of brain ammonia and glutamine were changed by electrical stimulation applied through skin receptors without evoking overt seizures.

In our experiments the ammonia values obtained by the freezing technique were somewhat higher than those obtained by RICHTER and DAWSON (1948). The reason for this may be that the body weight of the animals used was greater than that previously used by workers. It was necessary to use larger animals when applying the techniques of conditioned reflexes to rats. DAWSON (1950) has also reported higher values than those obtained previously (RICHTER and DAWSON, 1948). The glutamine concentration in the rat brain obtained by the method of hydrolysis with 10% trichloroacetic acid was lower than that found by hydrolysis with half-saturated KOH. Since the present values reported for ammonia and glutamine were obtained consistently, changes were always considered in comparison with figures obtained for resting controls.

The brain ammonia level obtained in rats stimulated for 5 sec, either by electrical stimulation or by the conditioned stimulus, was significantly higher than that of the resting controls. A stimulation of longer duration evoked no change, and anaesthesia resulted in a lowering of the brain ammonia level, as already reported by VLADIMIROV (1953).

The maximum brain ammonia level obtained after 5 sec stimulation returned to the normal level within 60 sec after stimulation. However, within 120 min after the first stimulation, a second electrical stimulation for 5 sec did not have any effect on the brain ammonia, although the ammonia level had already returned nearly to normal. It may be concluded that the processes concerned with ammonia metabolism in the rat brain were changed, and that the glutamine-synthesizing system was activated for 120 min after a 5 sec stimulation.

This conclusion is supported by the fact that the glutamine concentration was increased, without any increase in the ammonia level, by the continuous electrical stimulation for 30 min or by the intraperitoneal injection of NH_4Cl in doses as high as 200 mg/kg. The ammonia liberated in the brain would be rapidly fixed by combination with glutamic acid, and the glutamine formed would be metabolized further.

This view is supported by the finding that a rise in the brain ammonia level after electrical stimulation for 5 sec was suppressed by a previous injection of a small amount of NH_4Cl. The effect of injecting NH_4Cl might be attributed to the action of ammonium ions themselves, since it is difficult to assume that such a small dose of ammonium salt would produce a significant acidosis.

The duration of the suppression of the increase in brain ammonia resulting from a second stimulus after electrical stimulation or after the injection of NH_4Cl, was

significantly shortened in rats repeatedly stimulated with electric current or in animals repeatedly injected with small amounts of NH_4Cl. These findings suggest that the recovery processes from the disturbance in the ammonia-glutamine system in the brain caused by liberated ammonia are conditioned and promoted by the action of ammonium ions themselves.

It could be assumed that there is a dynamic equilibrium in the ammonia-glutamine system in the brain; if this is disturbed by the liberation of free ammonia, the ammonia would be removed by glutamine synthesis, which would be stimulated by the increased ammonia level.

On the basis of the present experiments, it would be difficult to distinguish chemically between the two physiological states characterized by excitatory and by inhibitory processes, although VRBA (1955*a*) has advocated a biochemical cycle of excitation and inhibition according to PAVLOV's theory, related to catabolic and anabolic processes involving the ammonia metabolism.

SUMMARY

(1) Ammonia levels in the rat brain, determined by the freezing technique in liquid air, were increased significantly by 5 sec electrical stimulation, and were not affected by stimulation of a duration longer than 5 sec. The level of ammonia was increased also by a conditioned stimulus (light) for 5 sec. Increase of glutamine content by stimulation was detected only when the stimulus was applied continuously for 30 min.

(2) Within 120 min after the first stimulation for 5 sec, a second stimulation did not cause an increase in the ammonia content, but after 150 min a second stimulation was effective again in increasing the brain ammonia level.

(3) Subcutaneous injection of ammonium chloride (20 mg/kg) suppressed for 60 min the increase in the ammonia concentration after 5 sec stimulation.

(4) In rats electrically stimulated for 5 sec ten times a day for 10 days, the brain ammonia was increased by a second stimulation applied 60 min after the first stimulation. Repeated injection of ammonium chloride (30 mg/kg) for 10 days, also shortened the period of suppression induced by electrical stimulation.

(5) The fate of ammonium ions liberated in the brain by stimulation under various conditions is discussed in relation to the physiological significance of the ammonia-forming and -binding (glutamine synthesis) systems.

Acknowledgements—The authors wish to express their thanks to Prof. T. HAYASHI for his interest and support. This work was aided by a grant from the Ministry of Education.

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CYTOCHROME OXIDASE AND SUCCINOXIDASE ACTIVITIES IN SPINAL GANGLION CELLS AND IN GLIAL CAPSULE CELLS

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(*Received* 28 *October* 1957)

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SINCE glial cells were first described by VIRCHOW (1846, 1854, 1867) most authors have believed that they constitute a protective, supporting and insulating component of the nervous tissue. However, other points of view have also been expressed. Based on comparative morphological observations, HOLMGREN (1901, 1904, 1914) thus came to the conclusion that the glial cells not only encapsulate the neuron with a thin envelope, but they also establish an intracellular contact with the nerve cell body. Material in the form of osmophilic granules was supplied to the nerve cell from the glial cells, and HOLMGREN suggested that the glial cells may have a nutritive function. That glial cells are somehow involved in the supply of metabolites to the nerve cells seems very probable indeed, when it is considered that metabolites on their way from the capillaries to the nerve cell bodies are first accessible to the glial cells. The fact that the glial cells are interposed between the capillaries and the nerve cells seems to favour the suggestion that they may be involved in nerve cell metabolism. A comparable arrangement exists in the spinal ganglia, in which the nerve cells are surrounded by a glial capsule. It is not surprising therefore, that several authors have suggested that glial cells may be of metabolic importance for the nerve cell.

If the possibility of a nutritive or metabolic function of the glial cells thus exists, it is quite another matter to devise experiments to demonstrate it. The function of the glial cells may be mainly to transport metabolites, so as to secure a sufficiently rapid supply to the nerve cells, but this would be very difficult to show. It may also be that there is a division of labour between glial and nerve cells, so that each type of cell is specialized for specific chemical activities. One possible way of obtaining information on this point is to study the activity of enzymes in the two types of cells. It is true that determinations of enzymic activity in general are of limited value because they give little information about the true activity in the cells and it is hard to meet this argument, but there is reason to believe that differences in the relative distribution of enzymes may indicate differences in metabolic activity.

Recently some papers have appeared in which determinations of various enzymes in nerve cell bodies and in glial cells have been described (ABOOD *et al.*, 1952, LOWRY *et al.*, 1954, LOWRY *et al.*, 1957, SNYDER *et al.*, 1952). The present paper is a further contribution, in which the relative distribution of the two mitochondrial enzymes, succinic dehydrogenase and cytochrome oxidase, has been studied. From our results it should thus be possible to get an idea about possible differences between

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the mitochondria in the two cell types. In the present investigation we have worked with spinal ganglia of the rabbit. We have compared the enzymic activity in isolated nerve cell bodies, in nerve cells lying in their glial capsules, and in isolated capsules.

EXPERIMENTAL

Material. Single nerve cells from rabbit spinal ganglia were used. The animals were killed by air injection. Immediately after death the spinal cord was exposed and a few cervical ganglia were dissected out. These were placed in about 5 ml of 0·25 M-sucrose solution at 2°. The cells were dissected just before the experiment, i.e. from 30 min to 6 hr after the animal had been killed. Two or three experiments, each taking 2–3 hr, were run in succession. This procedure seems to be safe in that the observed differences in enzyme activity were not related to the time the ganglia were stored in the ice box before the estimations. In one case two cells were assayed for succinoxidase 18 hr after the death of the animal, and the measured activities were within the same range as the other results.

Nerve cells (Fig. 1) were dissected free of the glial capsules in the following way: The ganglion was placed in a small dish in 0·25 M-sucrose solution, and freed from connective tissue with the aid of fine scissors and watchmaker's forceps. The ganglion was then cut through with a very sharp cataract knife. The cutting damaged many cells, but there were always a few capsules cut open with nerve cells not, or only slightly, damaged. By squeezing the cut surface very gently with the blunt side of the knife the cells could be forced out of the capsule. These cells float for some time in the sucrose solution but eventually they sink to the bottom. They stick easily to glass and to each other. Therefore all glass surfaces (dishes, pipettes) were coated with agar, as suggested by ZAJICEK and ZEUTHEN (1956). The cells were transferred to fresh sucrose solution with HOLTER braking pipettes.

Nerve cells and capsules (Fig. 2) were dissected from a small piece of spinal ganglion. First a thin piece of tissue (a few cells in thickness) was cut out with fine scissors and then one nerve cell and associated capsule was carefully freed from the surrounding capsule with two cataract knives. This can be done in less than 5 min. The procedure was carried out in 0·25 M-sucrose solution under a binocular microscope with about × 50 magnification. The system was transferred with a braking pipette to a fresh sucrose solution.

Capsules (Fig. 3) were obtained by first dissecting out a nerve cell plus capsule as described above. This was transferred to fresh sucrose solution and a cut was made through half the circumference of the capsule. The capsule was then expanded with two knives and the nerve cell removed. The nerve cell was generally damaged by this procedure, and therefore the nerve cells and the capsules studied here had different origins.

Only the largest cells were dissected (diameter: 75–100 μ), as we were interested in the distribution of enzymes between nerve and glial cells, rather than in the relation between cell size and enzyme activity. The diameter of the cells and systems were measured after dissection with a microscope at × 200 magnification. The accuracy of measurement, 0.5–1 μ, (smallest scale division = 4 μ) was sufficient for our experiments. The capsules were measured when they still contained the nerve cell, i.e. during the dissection procedure.

As mentioned above, the experimental material (cells, capsules, cells + capsules) were picked up and transferred with agar-coated braking pipettes. The dishes of sucrose solution were likewise agar-coated. It was not necessary to coat the dishes containing substrates (or the divers) as cytochrome diminishes the tendency of cells to stick to glass surfaces.

Estimations. The manometric method used in this work is that of ZAJICEK and ZEUTHEN (1956). Filling the divers was simplified by the fact that anaerobic conditions were not necessary. The cell to be assayed for one of the enzymes, was transferred to a drop of substrate (approx. 70 μl.) with a minimum amount of sucrose solution (not more than 0·1 μl.). If several cells had to be assayed at the same time (each cell in a separate diver) they were all placed in the same substrate drop. The cell was then sucked into a diver, which at that time was a part of a braking pipette, with about 0·2 μl. of substrate. The exact amount of substrate was determined after the experiment. The free end of the diver was first sealed with very small droplets of a soft paraffin and resin-wax mixture. Owing to its softness this wax could be put on the diver without heating. The small amount of soft wax served as indicator of the temperature during the sealing with a hard wax mixture; when the

soft wax was melted by being put near to the hot wax drop, the latter was allowed to cool until it could be brought to within 0·5 mm of the diver without causing the soft wax to melt. In this way overheating of the diver was avoided. The divers were made to float at a pressure as close to atmospheric as possible. The volumes of the substrate and of the air bubble in the diver were found after the experiment by weighing the diver charged and empty. The divers weighed 0·1–0·2 mg and had a gas volume of 0·05–0·1 μl.

All measurements were carried out at 37°. The divers, after being placed in the flotation vessels in the water bath, were left for 40 min for temperature equilibration, and then the readings were started. Readings were taken every 4–5 min for 80 min. Manometers and flotation vessels were as described by ZEUTHEN (1953), but the manometers were made from a capillary of greater internal diameter than that recommended by that author, 1 mm on the manometer scale corresponding approximately to 6 μl. Although this resulted in lower sensitivity, the manometers could be made shorter (35 cm) and were easier to use (Fig. 4). Usually two divers charged with substrate and experimental material, and one blank diver with substrate alone, were run simultaneously. All these divers were filled from the same substrate drop. The autoxidation in the blank was subtracted from the oxygen consumption in the experimental divers. In the cytochrome oxidase measurements the rate of autoxidation was high and it was therefore necessary to take into account any differences in substrate volume in the experimental and blank diver, when correcting for autoxidation. In the determinations of succinoxidase activity the blank oxidation rate was so low that it was unnecessary to correct for differences in substrate volume.

We are aware of the error in the measurements of cytochrome oxidase activity due to the fact that the autoxidation rate is affected by the presence of the enzyme preparation, i.e. the autoxidation rates in the experimental and blank divers were not the same (SLATER, 1949*a*). However there were big differences in cytochrome oxidase between morphologically identical cells. Therefore, the procedure generally recommended (various concentrations of enzyme, autoxidation rate extrapolated to zero concentration) could not be used here. Since autoxidation is slower in the presence of enzyme preparations, our results probably represent minimum values.

Calculations of oxygen consumption were made using ZEUTHEN's formula (1953) for measurements without CO_2 in the diver gas phase. The substrates used are listed below (all values representing final concentrations).

Substrates for cytochrome oxidase (SLATER, 1949*a*, POTTER, 1949):

(A) With sodium ascorbate as reducing agent: Phosphate buffer, pH 7·4, 0·0375 M; cytochrome *c* (Sigma) 10^{-4} M; Na ascorbate 0·0125 M; $AlCl_3$ 5×10^{-4} M.

(B) With *p*-phenylenediamine as reducing agent: Phosphate buffer, pH 7·4, 0·043 M; cytochrome *c* $1{\cdot}2 \times 10^{-4}$ M; *p*-phenylenediamine 0·043 M; $AlCl_3$ $5{\cdot}7 \times 10^{-4}$ M.

Substrate for succinoxidase (POTTER, 1949): Phosphate buffer, pH 7·4, 0·0375 M; cytochrome *c* 10^{-4} M; Na succinate 0·025 M; $AlCl_3$ 5×10^{-4} M; $MgCl_2$ 5×10^{-4} M.

Substrate for succinic dehydrogenase (SLATER, 1949*b*): Phosphate buffer, pH 7·4, 0·075 M; Na succinate 0·025 M; methylene blue 0·0009 M; KCN, neutralized, 0·009 M.

The flotation media used for the divers were of the same composition as the respective substrates, except that cytochrome *c* and methylene blue were omitted, and sodium ascorbate and *p*-phenylenediamine were replaced by isotonic concentrations of sodium chloride.

To avoid changes in the KCN concentration, the experiments in which KCN-containing solutions were used (succinic dehydrogenase) were not allowed to go on for more than 2 hr. Because of technical difficulties we did not try phenazine methosulphate for succinic dehydrogenase determination (SINGER and KEARNEY, 1957). Oxidation of sodium ascorbate and *p*-phenylenediamine without added cytochrome *c* in the substrate was measured only in divers of standard size, with ganglion pieces containing about fifty cells + capsules. Oxidation of succinate without added cytochrome *c* was measured both in divers of standard size and in the micro-divers used throughout this work. In these experiments we used substrates of the same composition as those for enzyme assays, except that cytochrome *c* was omitted.

RESULTS

In Table 1 the results obtained from cytochrome oxidase determinations are listed. The values for cells, capsules, and cells + capsules, respectively, are arranged into two groups according to the reducing substance. In each group the values are listed

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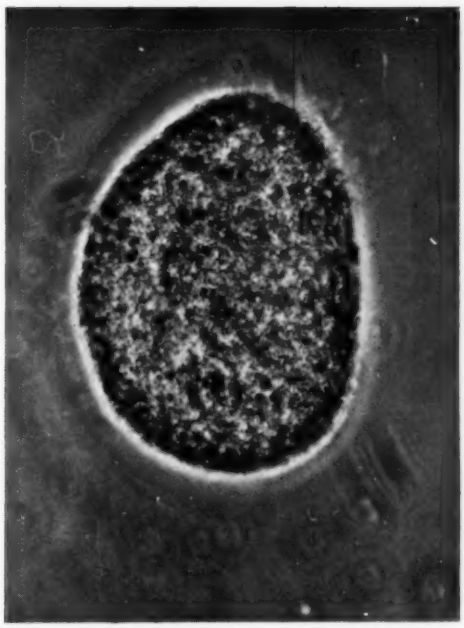

FIG. 1. Dissected spinal ganglion cell, rabbit, free from the capsule, photographed in a phase contrast microscope. Magnification 500×, objective 20×, num. ap. 0.45.

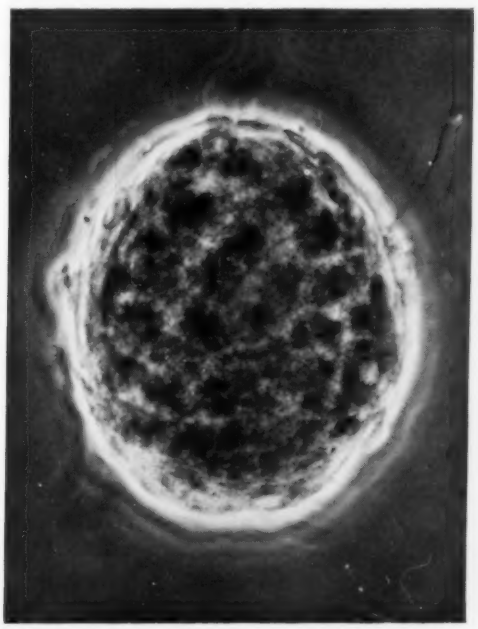

FIG. 2. Dissected spinal ganglion cell, rabbit, enveloped by its capsule with glial cells.

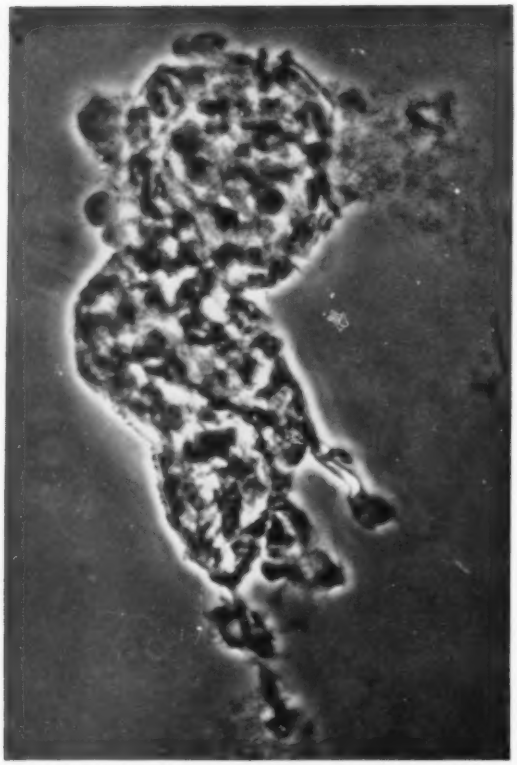

FIG. 3. Dissected capsule containing satellite glial cells.

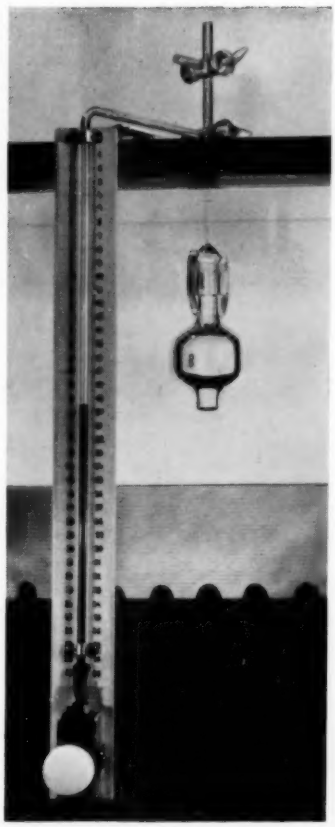

FIG. 4. Manometer and flotation vessel.

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TABLE 1.—CYTOCHROME OXIDASE ACTIVITIES IN SINGLE NERVE CELL BODIES, GLIAL CAPSULES, AND CELL BODIES WITH CAPSULES FROM RABBIT SPINAL GANGLIA
(Activities expressed as oxygen consumption in μl./cell/hr at 37°. Substrates: see text)

Cell diameter (μ)		Oxygen consumption (μl./cell/hr × 10^4)	Reducing agent
Nerve cell bodies	75 × 68	13·6	sodium ascorbate
	75 × 62	10·0	
	62 × 55	6·7	
	82 × 82	4·0	
	62 × 62	2·8	
	82 × 76	0	
	82 × 70	0	
	75 × 69	0	
	96 × 82	8·6	*p*-phenylenediamine
	79 × 65	5·7	
	82 × 79	3·7	
	82 × 62	3·4	
	56 × 56	0	
		Mean 4·5	
Glial capsules	—	1·9	sodium ascorbate
	76 × 72	1·0	
	82 × 68	0	
	80 × 68	0	
	88 × 76	4·9	*p*-phenylenediamine
	64 × 64	3·4	
	—	2·3	
	76 × 68	1·6	
	72 × 72	1·1	
	—	0	
		Mean 1·6	
Nerve cell bodies + glial capsules	76 × 70	9·6	sodium ascorbate
	70 × 62	7·3	
	96 × 82	4·2	
	95 × 82	3·9	
	90 × 75	3·0	
	82 × 75	2·5	
	82 × 76	0	
	96 × 82	12·7	*p*-phenylenediamine
	96 × 90	9·8	
	82 × 76	7·9	
	84 × 76	2·0	
	88 × 76	0·9	
		Mean 5·3	

in decreasing order. It will be noticed that there is no essential difference between the values obtained with the two reducing agents: but there is an enormous variation in the values in each group. As mentioned above, we believe that one of the reasons is the error introduced by the autoxidation correction. The autoxidation rates determined by us are probably too high, and the values of zero listed in the table may represent cases in which the autoxidation rate in the experimental divers was very low. The fact that the sum of the mean values for cells and for capsules agree rather well with the mean value for cells + capsules speaks in favour of the reliability of the results.

In Table 2 are listed the results obtained in succinoxidase determinations. It will be noticed that no values of zero were observed, but still the variation is quite large. We do not offer any explanation of this, but we would like to point out that a similar variation has been observed in determinations of acetylcholine esterase in single nerve cells (GIACOBINI, 1957). Also the results obtained with staining techniques show that differences in enzymic activity between different cells of the same tissue—as found in our experiments—seem to be of more general occurrence (SCHUMACHER, 1957). It will be noticed that the sum of the means for cells and for capsules agrees with the one for cells + capsules, but in view of the large individual variation the close agreement must be coincidental. Except for the nerve cells, the values are higher than for cytochrome oxidase. This observation may partly reflect the error in the cytochrome oxidase determinations, but it cannot be explained by this alone. It will be noticed from the table that addition of cytochrome is essential, even though a certain oxygen uptake may be found without the addition of cytochrome. The same holds for ascorbic acid and *p*-phenylenediamine oxidation. These results are not listed in the tables.

Some attempts to determine the succinic dehydrogenase activity are shown in Table 3. One would expect lower values with this method, since methylene blue is a less effective hydrogen carrier than cytochrome. On the other hand, the ratio between the means obtained with the two methods ought to be the same. As will be seen, this does not hold, the main reason being probably the very low activities and the small number of determinations.

DISCUSSION

The results show that the absolute activity of cytochrome oxidase in the nerve cell is higher than in the glial capsule, whereas the opposite is true for succinoxidase. For a real comparison, however, it would be important to know the activities per unit volume. We can at the moment only make an estimation of the relative volumes of nerve cells and of the glial capsules. On the basis of the photograph shown in Fig. 2, and assuming that the shapes of the capsule and the nerve cell, respectively are spheres having a diameter intermediate between those measured on the picture, we have calculated that the volume of the nerve cell is $4{\cdot}9 \times 10^5\ \mu^3$, and of the glial capsule $0{\cdot}9 \times 10^5\ \mu^3$. The volume of the nerve cell is thus five times greater than that of the glial capsule. The cytochrome oxidase activity per unit volume is thus $4{\cdot}5/5=0{\cdot}9$ in the nerve cell as compared with 1·6 in the glial capsule. For succinoxidase the corresponding numbers are $3{\cdot}5/5=0{\cdot}7$ and 5·1. Thus both enzymes exhibit higher activities per unit volume in the glial capsule, but the ratio for succinoxidase activities is seven as compared with about two for cytochrome oxidase.

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TABLE 2.—SUCCINOXIDASE ACTIVITIES IN SINGLE NERVE CELL BODIES, GLIAL CAPSULES, AND NERVE CELL BODIES WITH CAPSULES FROM RABBIT SPINAL GANGLIA
(Activities expressed as oxygen consumption in μl./cell/hour at 37°. Substrate: see text)

	Cell diameter (μ)	Oxygen consumption (μl./cell/hr × 10^4)	
Nerve cell bodies	82 × 82	6·8	
	82 × 68	4·8	
	68 × 68	4·7	
	70 × 62	4·0	
	—	3·6	
	—	3·3	
	70 × 60	3·0	
	75 × 62	2·8	
	82 × 75	2·7	
	76 × 62	2·4	
	—	2·3	
	70 × 70	2·2	
	—	1·6	
		Mean 3·4	
	80 × 80	5·1	no added cytochrome
	78 × 74	1·7	no added cytochrome
	74 × 68	0	no added cytochrome
Glial capsules	90 × 72	9·4	
	100 × 98	8·7	
	88 × 80	5·8	
	110 × 94	5·1	
	96 × 90	4·2	
	95 × 82	4·0	
	100 × 90	3·5	
	—	1·7	
	—		
		Mean 5·1	
	96 × 90	0·5	no added cytochrome
	82 × 78	0	no added cytochrome
	78 × 74	0	no added cytochrome
Nerve cell bodies + glial capsules	86 × 82	17·5	
	84 × 84	11·7	
	—	10·8	
	95 × 82	10·6	
	82 × 75	8·8	
	—	7·9	
	90 × 75	7·4	
	76 × 76	4·4	
	62 × 60	3·5	
	78 × 72	2·3	
		Mean 8·5	

TABLE 3.—SUCCINIC DEHYDROGENASE ACTIVITY IN SINGLE NERVE CELL BODIES, GLIAL CAPSULES, AND NERVE CELL BODIES WITH CAPSULES FROM RABBIT SPINAL GANGLIA

(Activities expressed as oxygen consumption in μl./cell/hr at 37°. Substrate: see text)

	Cell diameter (μ)	Oxygen consumption (μl./cell/hr $\times 10^4$)
Nerve cell bodies	82 × 70	2·6
	96 × 75	2·4
	76 × 70	2·0
	76 × 70	1·5
	90 × 70	0·6
	56 × 50	0·6
		Mean 1·6
Glial capsules	80 × 80	1·1
	78 × 68	0·6
	70 × 68	0·1
	80 × 72	0
		Mean 0·5
Nerve cell bodies + glial capsules	82 × 82	5·0
	75 × 69	2·5
	96 × 75	2·2
	90 × 75	1·8
	89 × 56	1·4
	70 × 70	0·9
	70 × 56	0·4
		Mean 2·0

We realize that the values obtained probably do not represent the enzymic activities within the living cell. In reactions of cell metabolism enzymic activities are seldom the limiting factor, and it would therefore be difficult to conclude from the findings anything about the relative contributions of nerve cell and its glial capsule in energy production for example. However, as far as we may consider the observed differences as real, they indicate a certain specialization, i.e. division of labour between the two types of cells. It seems premature to speculate upon what this specialization involves.

SUMMARY

Using the microdiver technique of ZAJICEK and ZEUTHEN, the activities of cytochrome oxidase and succinoxidase were measured in single nerve cells with or without their capsules containing the glia cells, and in single capsules from spinal ganglia of rabbits. There are large individual differences in enzymic activity between cells of the same size; mean values for cytochrome oxidase (expressed as oxygen consumption per hour at 37°) are: $4{\cdot}5 \times 10^{-4}$ μl per nerve cell body, $1{\cdot}6 \times 10^{-4}$ μl

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per capsule and $5{\cdot}3 \times 10^{-4}$ μl per nerve cell + capsule. Mean values for succinoxidase activities, expressed as oxygen consumption per hour at 37°, are: $3{\cdot}4 \times 10^{-4}$ μl per nerve cell, $5{\cdot}1 \times 10^{-4}$ μl per capsule and $8{\cdot}5 \times 10^{-4}$ μl per nerve cell + capsule.

Acknowledgements—We should like to express our gratitude to Prof. O. LINDBERG for his kind criticism of the manuscript. The present work has been supported by grants from The Swedish Medical Research Council.

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Journal of Neurochemistry, 1958, Vol. 2, pp. 312–317. Pergamon Press Ltd., London

CHANGES IN α-KETO ACID LEVELS IN RABBIT BLOOD AFTER CHLORPROMAZINE, RESERPINE AND SODIUM PENTOBARBITAL ADMINISTRATION*

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(*Received* 7 *October* 1957)

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THE inhibitory properties of chlorpromazine (ABOOD, 1955) and reserpine (BERNSOHN, NAMAJUSKA and BOSHES, 1956) on some of the enzyme systems involved in glucose degradation have been demonstrated in brain mitochondria. These drugs inhibit adenosine triphosphatase (ATP-ase) and cytochrome oxidase (CENTURY and HORWITT, 1956), as well as α-ketoglutarate and succinate degradation, and uncouple oxidative phosphorylation (BERNSOHN, NAMAJUSKA and COCHRANE, 1956). The findings have suggested that a common mechanism of action of the two tranquillizing drugs may reside in their inhibiting electron transport, and/or acting on ATP-ase, thereby creating a deficit in adenylate acceptors, so that high-energy phosphate transfer is impeded. In either case, it should be possible to demonstrate an alteration in the level of some of the metabolites involved in these inhibitory processes. Thus, EL HAWARY (1955) has shown that the administration of specific enzyme inhibitors such as arsenite, iodoacetate, malonate, fluoride, and maleate leads to increased blood α-keto acid levels. If the findings on glucose metabolism *in vitro* were the basis of the pharmacological action of the drugs, it would appear reasonable that *in vivo* manifestations of the inhibitory effects might be reflected in changes in the levels of some glucose intermediates after drug treatment.

Because of the central role of the α-keto acids, and pyruvate in particular, in carbohydrate metabolism, a study of these substances was undertaken. To determine whether the observed changes were specific for the tranquillizing drugs, studies were made using sodium pentobarbital in addition to saline controls. Initial attempts to study the levels of these metabolites in brain as well as in blood were abandoned when it was found that the analytical methods presented some difficulties unique to brain tissue (unpublished observations).

MATERIALS AND METHODS

White albino rabbits, weighing 2–3 kg, were fasted for 16–18 hr prior to, and during the experimental period. They were tied to an animal board to prevent struggling and after withdrawal of fasting blood samples, injection of the drug was given at zero time. At appropriate intervals, 5–7 ml of blood were withdrawn by cardiac puncture using a 22-gauge needle. The blood was transferred from the syringe into a tared tube containing 25 ml of 5% metaphosphoric acid, and the weight of blood was obtained by difference. A slightly modified chromatographic procedure of EL HAWARY and THOMPSON (1953) was used for the determination of the keto acids. The extracted and dried 2:4-dinitrophenylhydrazones of the keto acids were redissolved in a known amount of

* This work was aided by a grant from the Illinois Mental Health Fund.

ethyl acetate before placing on paper strips. Descending chromatography was employed. After elution from the paper and colour development, solution density was read in a Beckmann DU spectrophotometer at 440 mμ with the aid of a microcuvette. Glucose was determined by the method of NELSON (1944).

All compounds used were injected subcutaneously under the abdominal skin. The dose of chlorpromazine* was 50 mg per rabbit, reserpine† 1 mg/kg body wt., and sodium pentobarbital 35 mg/kg body wt. The latter dosage was insufficient to render the animal unconscious. In the controls, 1 ml of isotonic saline was injected in a similar manner.

Subsequent experiments were performed to determine the minimal drug level required to elicit a response in blood α-keto acid level. The drugs were also administered subcutaneously at levels of 8, 5 and 4 mg/kg of chlorpromazine, and 0·5 and 0·2 mg/kg of reserpine per animal. Results of the data obtained are the observations on 4–5 animals in each group.

RESULTS

Standard amounts of α-ketoglutaric and pyruvic acids which were added to the solution of metaphosphoric acid at the same time as the blood showed recoveries of 104 per cent and 87 per cent for the two acids respectively (Table 1). The slightly

TABLE 1.—RECOVERY OF STANDARD α-KETOGLUTARIC AND PYRUVIC ACIDS FROM RABBIT BLOOD

	α-ketoglutaric acid				Pyruvic acid			
Expt.	Sample (μg)	Added (μg)	Recovered (μg)	(%)	Sample (μg)	Added (μg)	Recovered (μg)	(%)
1	0·6	2·8	3·7	109	5·4	2·8	7·1	87
2	1·0	2·8	3·7	103	5·2	2·8	6·6	82
3	1·7	5·6	7·4	101	10·6	5·6	14·8	91
4	1·9	5·6	7·7	103	11·4	5·6	15·0	88
Average				104				87

high recovery values for α-ketoglutaric acid may be due to the low R_F value and its proximity to the origin. While EL HAWARY and THOMPSON (1953) did not report recoveries of standards in their work, they did compare their results with those obtained by the FRIEDEMANN and HAUGEN (1943) method. Their chromatographic technique gave values of 84 per cent of the total α-keto acids, obtained by the colorimetric determination, and this compares favourably with the recovery of 87 per cent of pyruvic acid reported here. Since the normal fasting α-ketoglutarate level is only about 10 per cent of the total α-keto acids (see Table 2), the higher recoveries of that acid would not materially alter the overall picture.

The changes in the blood levels of α-ketoglutarate and pyruvate after the administration of the three compounds under study are shown in Table 2. While the α-keto acid levels varied from one animal to another, the response to the drugs calculated

* Chlorpromazine was kindly donated by Smith, Kline & French Laboratories, Philadelphia, Pennsylvania.

† Reserpine was kindly donated by the Research Laboratories, CIBA Pharmaceutical Co., Summit, New Jersey.

as a percentage decrease from the zero hour level was remarkably constant. Some glucose values are also reported, and the pyruvic acid values are presented together with the standard error of the mean. It can be seen that changes in blood glucose and blood α-ketoglutarate are minimal and are not significantly altered during the

TABLE 2.—EFFECT OF PENTOBARBITAL (SODIUM), CHLORPROMAZINE AND RESERPINE ON α-KETO ACIDS IN RABBIT BLOOD

Compound	Time (hr)	Glucose (mg/100 g)	α-Keto glutarate (mg/100 g)	Pyruvate (mg/100 g)	(S.E.M.)
Saline	0	85	0·22	2·07	±0·18
	2	94	0·32	1·98	±0·67
	3	102	0·26	1·94	±0·30
	24	90	0·22	1·84	±0·41
Pentobarbital	0	95	0·18	2·20	±0·17
	3	93	0·16	0·51	±0·05
	6		0·16	1·19	±0·11
	10		0·16	2·12	±0·17
	24	108	0·18	2·20	±0·22
Chlorpromazine	0	96	0·20	1·88	±0·42
	1		0·22	1·47	±0·48
	2	94	0·25	0·90	±0·18
	3	87	0·23	0·68	±0·17
	5		0·24	0·68	±0·10
	8		0·27	0·58	±0·21
	24		0·22	0·75	±0·43
Reserpine	0	79	0·19	2·23	±0·34
	2	94		2·25	±0·32
	6			1·49	±0·18
	12		0·24	0·94	±0·32
	20		0·23	0·67	±0·32
	24	107	0·20	0·51	±0·13
	30		0·21	0·52	±0·09

Dosage: Pentobarbital, 35 mg/kg; chlorpromazine, 50 mg/rabbit; reserpine, 1 mg/kg. All compounds were injected subcutaneously. Values are averages of 4–5 animals.

test period by either saline or the drugs under study. However, there are marked changes in pyruvate levels, and these changes vary depending on the drug employed.

With pentobarbital, the pyruvate level falls from 2·20 mg/100 g to 0·51 mg/100 g 3 hr after the injection of the drug. The blood pyruvate then starts to rise, and after 10 hr reaches the initial value. In contrast to this, after chlorpromazine administration

the pyruvate falls from 1·88 mg/100 g to a level of 0·68 mg/100 g after 3 hr; and after 8 hr the blood pyruvate level is still depressed to 0·58 mg/100 g. Only a slight elevation from this low level is evident 24 hr after the drug has been given, the value rising to 0·75 mg/100 g.

The picture with reserpine differs from the other two drugs in the rate of blood pyruvate decrease. No change is evident 2 hr after the drug has been administered,

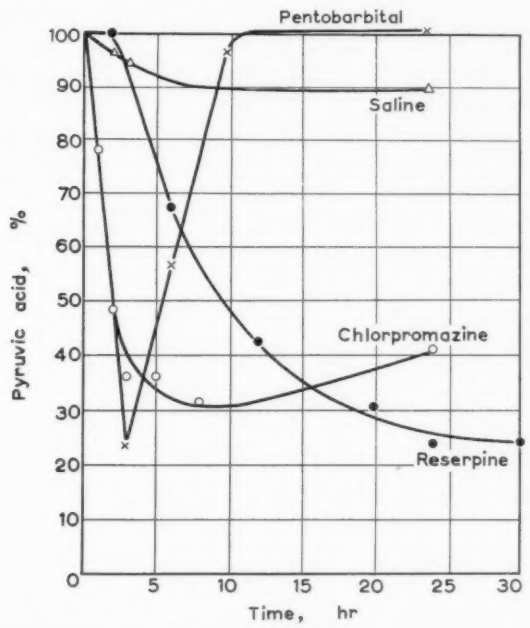

FIG. 1. Blood pyruvic acid changes in rabbits after subcutaneous injection of saline, pentobarbital (sodium), chlorpromazine and reserpine.

and only after 6 hr has the blood pyruvate level shown any decrease from 2·23 mg/100g to 1·49 mg/100 g. This relatively slow decline is maintained, reaching a value of 0·94 mg/100 g after 12 hr; and only after 24 hr is a minimum value of 0·51 mg/100 g reached, with no recovery after 30 hr.

Thus while the three drugs all reduce the blood pyruvate level to approximately the same value, the time required to produce this low level and the rate of recovery to normal value are characteristic for each drug. These time-pyruvate curves are shown in Fig. 1, where the time is plotted against the percentage of the fasting blood pyruvate level for each compound. The varying types of curves obtained for the different compounds are evident.

The dosage of chlorpromazine and reserpine was then reduced, to determine the minimum effective dose. Chlorpromazine at an 8 mg/kg level produced a blood pyruvate level of 41 per cent of the basal value at 6 hr, at 5 mg/kg the value was 45 per cent and at 4 mg/kg 77 per cent of the basal pyruvate level was attained. The pyruvate levels were higher at the end of 24 hr, as in the previous experiments.

When the dose of reserpine was reduced to 0·5 mg/kg or 0·2 mg/kg, the pyruvate

TABLE 3.—EFFECT OF DRUG DOSAGE ON PYRUVATE LEVELS IN RABBIT BLOOD

Compound	Time (hr)	Glucose (mg/100 g)	Pyruvate (mg/100 g)	Pyruvate (% of pre-injection level)
Chlorpromazine 8 mg/kg	0	110	1·87	—
	6	106	0·77	41 ± 11·8*
	24	94	0·96	51 ± 21·0
5 mg/kg	0	85	1·79	—
	6	92	0·81	45 ± 8·6
	24	100	1·09	61 ± 10·3
4 mg/kg	0	92	1·60	—
	6	102	1·23	77 ± 8·4
	24	104	1·30	82 ± 10·6
Reserpine 0·5 mg/kg	0	86	2·17	—
	24	97	1·17	54 ± 12·8
0·2 mg/kg	0	85	2·02	—
	24	97	1·56	77 ± 10·8

* S.E.M.

corresponded to 54 per cent or 77 per cent respectively of the fasting pyruvate level. Thus, it would seem that the effective dosage of chlorpromazine and reserpine required to produce a fall in blood pyruvate was between 2 and 5 mg/kg for chlorpromazine, and between 0·2 and 0·5 mg/kg for reserpine, when the drugs were administered subcutaneously.

DISCUSSION

A fall in blood pyruvate levels after pentobarbital administration has been reported by GOODWIN and WILLIAMS (1952). However, a decrease in α-ketoglutarate was observed in their study, in contradistinction to the results obtained in this report. Since the two procedures differed in several respects, the discrepancy in the findings may be due to some variable in experimental technique. It might be expected that a fall in blood pyruvate levels would also lead to a drop in blood α-ketoglutarate levels because of the equilibria of these two metabolites in the citric acid cycle. However, a diminution of α-ketoglutarate *via* pyruvate could be compensated for by a shift in the transaminase equilibrium tending to maintain normal levels of the former intermediate.

The decrease in blood pyruvate levels after drug administration can reflect either an over-utilization or under-production of this metabolite. Since sedation would be incompatible with an accelerated metabolism, and since other changes observed during sedation indicate 'a picture not of exhaustion, but of non-utilization of the sources of energy' (RICHTER, 1952), it must be considered that the low pyruvate

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levels reflect a diminution in the production of this substance. The absence of hypoglycemia during the experimental period would exclude a lack of available glucose as a causative factor. It would appear from these data that the degradation of glucose is inhibited somewhere between the glucose ⇌ pyruvate series of reactions.

The decreased pyruvate production may result from a diminished glucose dissimulation due to a reduction in energy requirements during sedation, or another explanation may be that the compounds act by specifically inhibiting one or more of the reaction steps involved in glucose degradation. Since chlorpromazine and reserpine have been shown to be ATP-ase inhibitors *in vitro*, this could lead to a decrease in AMP and ADP as adenylate acceptors for high-energy phosphate bond transfer, causing a general deceleration of the rate of glucose metabolism. The observations of GRENELL, MENDELSOHN and MCELROY (1955) of high ATP levels in brain after chlorpromazine administration, and the work of BAIN (1956) who observed that depressants and chlorpromazine inhibit the incorporation of labelled phosphate into brain nucleotides, tend to support this concept.

That the three drugs have the same effect in reducing blood pyruvate does not invalidate this hypothesis as a mechanism of action of the tranquillizing drugs since the response pattern differs. Pentobarbital gives an abrupt decrease in pyruvate with relatively rapid recovery to normal levels. The recovery of fasting blood pyruvate levels is slower with chlorpromazine, and reserpine shows a slow diminution of this metabolite with no recovery at 30 hr post-injection. That this differing response is not due to differences in absorption is evident when the onset of sedation is compared. That mere immobilization due to sedation is not the cause of the decreased pyruvate levels, is indicated by the results obtained with the tranquillizing compounds, where the low values persisted for hours subsequent to the recovery of the animals from the effects of the drugs. Whether the differences in pyruvate response can account for the variability in the pharmacological properties of the tranquillizing and depressant drugs remains to be ascertained.

SUMMARY

The effect of chlorpromazine and reserpine on α-keto acid levels in rabbit blood has been determined, and compared with results obtained after sodium pentobarbital administration. While α-ketoglutaric acid was not significantly altered, a marked decrease in pyruvic acid was obtained, with a time-course characteristic for the drug employed. The significance of the results in terms of mechanism of drug action and carbohydrate metabolism is discussed.

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Journal of Neurochemistry, 1958, Vol. 2, pp. 318–325. Pergamon Press Ltd., London

METABOLIC STUDIES ON PHOSPHOLIPIDS AND NUCLEIC ACID OF FROG NERVE DURING EXCITATION*

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(*Received* 15 *July* 1957)

INTRODUCTION

THE application of isotopic tracers opened up innumerable avenues of approach to the problem of the relation of chemistry to neural function, while at the same time helping to dispel many misconceptions based on a lack of sound experimental observations. It has been presumed for over a quarter of a century that since respiratory activity increased during excitation of neural, as well as muscular tissue, the energetic machinery must turn over more rapidly. Evidence in support of this notion is completely lacking, while a formidable body of evidence is contrary (ABOOD and GOLDMAN, 1956; MOMMAERTS, 1955; MULLINS, 1954). After it had been established that phosphorylative activity, as measured by ^{32}P turnover of 'acid-soluble' components was, if anything, decreased during tetanization of frog sciatic nerves (ABOOD and GOLDMAN, 1956), it remained to be determined whether any of the 'acid insoluble' or structural phosphorylated compounds were behaving differently. In the course of investigating the phosphate turnover of the phospholipids and nucleic acid in amphibian nerve during excitation, an effort was made to determine any quantitative changes in various structural components. A number of interesting reports have recently appeared describing structural changes occurring in the NA† and protein constituents during activity of the cat brain (GEIGER *et al.*, 1956), nerve cell bodies (HYDÉN, 1951) and the nerve fibre (UNGAR *et al.*, 1957).

METHODS

The sciatic nerves of bullfrogs, with the surrounding connective tissue carefully removed, were incubated at 25° for 1 hr in a solution of phosphate-free frog's Ringer containing 100 μc of virtually carrier-free ^{32}P-orthophosphate/ml. It was essential that the ^{32}P-orthophosphate be used within 2 weeks after being prepared by the Oak Ridge National Laboratories, since the material contained many impurities that were found to be inhibitory to phosphorylation in large concentrations. After the nerves were washed three times in 100 ml of frog's Ringer solution at 0°, they were placed on a grid of silver wires mounted in a lucite box (tightly covered to maintain a saturated atmosphere) at 25°. Half of the nerves were kept as unstimulated controls, while the other half were stimulated with square wave pulses at a potential of 3 v, a frequency of 120 cyc/sec, and a pulse duration of 0·5 msec. The action potentials were recorded continuously with a cathode ray oscilloscope. After stimulation for 1 hr, during which time the action potential appeared normal, the nerves were removed and immersed for 3 min in three successive 100 ml portions of frog's P-free Ringer at 0°. Usually, two control or two stimulated nerves were used in a single determination. The average wet weight of two nerves was around 400 mg.

* This research was supported in part by a contract from the Office of Naval Research and in part by a grant from the Illinois Department of Public Welfare, Mental Health Fund.

† The following abbreviations are used: Nucleic acid (NA); ribonucleic acid (RNA); desoxyribonucleic acid (DNA); adenosinetriphosphate (ATP); orthophosphate (P).

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The nerves were homogenized in 6 ml of 1·5 N-perchloric acid at 0°, and the homogenate centrifuged in the homogenizer itself. In order to remove most of the acid-soluble phosphorylated intermediates, it was necessary to successively homogenize and sediment the residue four times with 10 ml portions of 0·5 N-perchloric acid at 0°.

Extraction of phospholipids and a phosphatide-peptide complex. A modification of a method of FOLCH *et al.* (1951) was employed. The washed nerve residue was homogenized in 10 ml of 3 : 1 chloroform-methanol (v/v) and allowed to stand at room temperature for 30 min. After removal of the lipid extract by centrifugation, the procedure was repeated and the extracts combined. To the chloroform-methanol extract was added 3·5 ml of water, whereupon the mixture was shaken vigorously in a glass-stoppered centrifuge tube for 2 min and then centrifuged at 0°. The mixture was separated by this procedure into three layers: a chloroform-methanol fraction, an aqueous-methanol fraction and an interfacial fluff. Separation of the three layers was accomplished by first removing the lower layer by suction and then carefully pouring off the remaining water-methanol layer while permitting the fluff to adhere to the sides of the centrifuge tube.

Characterization of the 'fluff' layer as a complex of polypeptides and phosphatides was based both on its solubility properties and certain chemical determinations. The fluff was soluble in certain organic solvents, such as chloroform–methanol and ether–alcohol (1 : 3 v/v). Apparently the peptides and lipids were complexed in so far as it was not possible to separate the two by repeated extraction with water. The reactivity of the material with ninhydrin increased gradually upon hydrolysis in N-hydrochloric acid at 100°. The acid hydrolysate was subjected to two-dimensional paper chromatography, using butanol : acetic acid : water (40 : 10 : 40 v/v) and 80% phenol. The detailed analysis of this fraction will be reported later, but it contained most of the amino acids present in the phosphatide–peptide fraction described by FOLCH (1952). In addition, the hydrolysate contained fatty acids and orthophosphate. More detailed analysis of this fraction is in progress at present. Subsequent analysis revealed that the chloroform–methanol layer contained phospholipids, and the aqueous methanol fraction consisted of polypeptides and a few phospholipid fragments.

Chromatographic analysis of phosphorylated amino acids and bases. The chloroform–methano-fraction and the interfacial fluff were transferred to vials, dried, sealed after adding 5 ml of 6 N-hydrol chloric acid, and hydrolysed at 100° for 1 hr. After removal of the fatty acids and remaining phospholipids by repeated ether and chloroform extraction, the hydrolysates were concentrated *in vacuo* to a volume of about 1 ml, transferred quantitatively to a 2 ml conical centrifuge tube and brought to complete dryness. To the residue was added 0·1 ml of 0·1 N-HCl, and the solution was ready for paper chromatography.

Two dimensional paper chromatography was employed for the separation of the phosphorylated constituents of the hydrolysates. The first solvent consisted of butanol : acetic acid : water (40 : 10 : 40 v/v) and the second of 80% phenol containing 0·01% sodium versenate. The phosphorylated amino derivatives (e.g. phosphorylethanolamine) and amino acids were identified by spraying with 0·1% ninhydrin in butanol, according to CONSDEN *et al.* (1944). Phosphorylated compounds were identified by spraying with a solution of 0·2% ammonium molybdate in 0·7% perchloric acid, heating at 80° for 5 min, and then exposing to an atmosphere saturated with hydrogen sulphide fumes for about 30 sec. Radioactivity was determined by cutting out the spots and counting them as such in a conventional scaler.

Ion exchange chromatography of NA nucleotides. Upon removal of the lipid fraction, the remaining acid-insoluble residue was suspended in 3·0 ml of 0·3 N-potassium hydroxide and incubated at 37° for 18 hr to hydrolyse the RNA to mononucleotides (DAVIDSON and SMELLIE, 1952). The mixture was cooled in an ice bath and centrifuged in the cold. After removal of the potassium by neutralization with 2 N-perchloric acid at 0°, the clear supernatant, which contained the mononucleotides of RNA, was ready for chromatography. Removal of the DNA from the insoluble residue was accomplished by hydrolysis in 0·5 N-perchloric acid at 90° for 10 min. The DNA hydrolysate was then neutralized with potassium hydroxide and the potassium perchlorate was removed by centrifugation. After evaporation to dryness at 90°, 0·5 ml of concentrated perchloric acid was added, and the solution hydrolysed in a boiling water bath for 1 hr, a procedure yielding free purine and pyrimidine bases (MARSHAK and VOGEL, 1951). In those instances where only total NA was to be determined, the acid-insoluble residue was extracted with hot perchloric acid, as described above, without prior removal of RNA with dilute alkali.

The general technique for the preparation of columns for ion exchange chromatography has been

described elsewhere (ABOOD and GOLDMAN, 1956). For the separation of nucleotides, Dowex-1 × 8 (100–200 mesh), as the formate, with a continuous mixing device was employed. The mixing flask originally contained 500 ml of distilled water, while the upper reservoir contained 4 N-formic acid. Elution was at the rate of 5 ml per 10 min per tube, with the aid of an automatic fraction collector. Separation of the nucleotides was accomplished in much the same order as described by VOLKIN *et al.* (1951), where discontinuous elution was performed with hydrochloric acid. Purine and pyrimidine bases were separated on a cation exchange column (8 × 0·8 cm) using Dowex-50 × 8 (100–200 mesh) resin. A continuous mixing device was used for elution where the mixing flask originally contained 200 ml of water and the upper reservoir 3 N-hydrochloric acid, after a modification of a technique of COHN (1950). Elution was accomplished at the rate of 2·0 ml per 2 min per tube. Identification of the bases and nucleotides was through measurement of absorbance at the appropriate wavelengths with the DU ultraviolet Beckmann spectrophotometer. Further identification of the bases was accomplished with the aid of paper chromatography, using butanol : acetic : water (40 : 10 : 10 v/v) as the developing solvent. Radioactivity of the nucleotides was determined on liquid samples with the conventional Geiger tube and scaler to a probable error of 2 per cent.

Separation of inositol phosphate. In the course of extracting RNA with 0·3 N-potassium hydroxide an extremely acid-resistant organic phosphate was simultaneously extracted which could be separated from the RNA nucleotides on a Dowex-1 column. After all the nucleotides and orthophosphate were eluted with increasing concentrations of formic acid, 1 N-hydrochloric acid was used for the elution of the organic phosphate. This fraction was subsequently identified as an inositol phosphate and contained approximately 2 moles of phosphate/mole of inositol. It was possible to separate adenylic acid from orthophosphate by drying the combined fractions, rechromatographing, and eluting with a technique described previously (ABOOD and GOLDMAN, 1956). In the course of elution a number of ninhydrin-reacting substances were found to be present. These substances, which were shown to be amino acids and polypeptides, had optical absorbance at the optimal wavelength (250–280 mμ) of the nucleotides. Since such substances were adequately separated from the nucleotides, they did not interfere with the quantitative estimation of the nucleotides. Inositol was identified microbiologically with *Schizosaccharomyces pombe**, as described by YARBROUGH and CLARK (1957), except that the phosphate was employed instead of free inositol.

RESULTS

Neither the DNA or RNA (difference between Tables 1 and 2) content of bullfrog sciatic nerves *in vitro* was altered significantly after electrical tetanization for 1 hr. The ratio of adenine + thymine to guanine + cytosine for nerve was 1·28. The molar ratio of cytosine : uracil : guanine : adenine : thymine was 1·00 : 0·89 : 0·90 : 1·18 : 0·41.

A 28% drop in the free amino acid content was observed after stimulation (Table 3). The phospholipid and phosphatide-peptide content did not appear to change significantly as determined by their total ninhydrin-reactive substances.

After 1 hr of stimulation, a slight decrease in the specific activity of the RNA nucleotides was observed. Cytidylic acid showed the greatest decrease (13 per cent) while uridylic and guanylic acid were essentially unchanged. No significant change was observed in the total amount of any of the nucleotides as a result of tetanization. The rate of turnover of ^{32}P of all the nucleotides of RNA appears to be approximately equal as adjudged by the specific activity. There is an indication, however, that adenylic acid has the highest rate of turnover.

Although the actual amount of inositol phosphate, the phosphatide-peptide complex, and remaining phospholipids did not change as a result of excitation, differences in their phosphate turnover were observed (Table 5). The specific activity of inositol phosphate and phospholipid was inhibited 12 per cent ($P < 0·01$) after stimulation for 1 hr, while the inhibition in the phosphatide-peptide was only slight

* The authors are indebted to Dr. F. M. CLARK for a culture of *Schizosaccharomyces pombe*.

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TABLE 1.—EFFECT OF ELECTRICAL EXCITATION ON DNA BASES OF FROG SCIATIC NERVE

Values are mean of four determinations agreeing within 7 per cent. All changes are non-significant. 'C' designates control nerves, and 'S' nerves stimulated for 1 hr.

DNA purine and pyrimidine bases			
		Amount (μmoles/g)	Change (%)
Cytosine	C	0·102	—
	S	0·098	−5
Thymine	C	0·128	—
	S	0·120	−7
Guanine	C	0·108	—
	S	0·115	6
Adenine	C	0·140	—
	S	0·136	−3

and non-significant. The specific activity of inositol phosphate was over three times greater than the phosphatide-peptide component, which in turn was about twice that of the phospholipid fraction.

DISCUSSION

The results of the present investigation are in conformity with the hypothesis that excitation of peripheral nerve is not accompanied by increased phosphorylation, but rather, in some instances, a decreased phosphorylation (ABOOD and GOLDMAN, 1956).

TABLE 2.—EFFECT OF ELECTRICAL EXCITATION OF BULLFROG SCIATIC NERVES ON PURINE AND PYRIMIDINE BASES OF RNA AND DNA

Values are expressed as mean ± standard deviation and units are μmoles/g wet weight of nerve. 'C' designates control and 'S' stimulated nerves. The number of experiments was 14.

Base		Amount (μmoles/g)	Change (%)
Cytosine	C	0·275 ± 0·05	—
	S	0·255 ± 0·03	−7
Uracil	C	0·245 ± 0·04	—
	S	0·234 ± 0·04	−4
Guanine	C	0·246 ± 0·05	—
	S	0·240 ± 0·05	−3
Adenine	C	0·325 ± 0·06	—
	S	0·304 ± 0·05	−7
Thymine	C	0·101 ± 0·05	—
	S	0·095 ± 0·03	−6

TABLE 3.—CHANGE IN FREE AND LIPID-CONTAINED AMINO ACIDS OF BULLFROG NERVE AFTER ELECTRICAL STIMULATION

Values are expressed as mean ± standard deviation. 'C' designates control nerves and 'S' nerves stimulated for 1 hr after previous soaking in ^{32}P.

Source		No. of experiments	Amount (μmoles/g)	Change (%)	P
Free amino acids	C	9	9·7 ± 1·5		
	S	9	7·0 ± 1·8	−28	0·001
Phospholipid (phosphorylated)	C	10	8·5 ± 0·9		
	S	10	8·0 ± 1·0	−6	non-sig.
Phosphatide-peptide complex	C	12	15·0 ± 1·0		
	S	12	14·5 ± 1·2	−3	non-sig.

TABLE 4.—EFFECT OF TETANIZATION ON ^{32}P TURNOVER IN NUCLEOTIDE COMPONENTS OF RNA FROM BULLFROG NERVES

The number of experiments was 12. None of the differences were statistically significant. 'C' designates control, and 'S' stimulated nerves.

Nucleotide		Amount (μmoles/g)	(counts/min)	Sp. act. (counts/min/μmole P)	Change (%)
Cytidine	C	0·200 ± 0·03	245 ± 45	1,265	
	S	0·205 ± 0·03	200 ± 56	980	−13
Uridylic	C	0·206 ± 0·03	270 ± 60	1,310	
	S	0·198 ± 0·02	255 ± 50	1,295	−3
Adenylic	C	0·195 ± 0·03	285 ± 70	1,460	
	S	0·200 ± 0·03	270 ± 52	1,340	−8
Guanylic	C	0·220 ± 0·03	285 ± 75	1,300	
	S	0·215 ± 0·04	270 ± 45	1,260	−4

TABLE 5.—EFFECT OF EXCITATION ON SPECIFIC ACTIVITY OF VARIOUS FRACTIONS OF BULLFROG SCIATIC NERVES

'C' designates control and 'S' nerves stimulated for 1 hr after previous soaking in ^{32}P for 1 hr.

		No. of experiments	P* (μmoles/g)	^{32}P (counts/min)	Sp. act. (counts/min/μmole P)	Inhib. (sp. act.) (%),
Inositol phosphate	C	8	0·55 ± 0·06	5,550	10,100	—
	S	8	0·52 ± 0·07	4,600	8,900	12†
Phosphatide-peptide complex	C	9	5·5 ± 0·4	17,500	3,160	—
	S	9	5·0 ± 0·4	15,000	3,000	5
Remaining phospholipids	C	12	18·0 ± 0·2	32,000	1,780	—
	S	12	17·8 ± 0·2	28,000	1,570	12†

* Mean ± standard deviation
† Significant at $P < 0·01$

Previously, it had been demonstrated that the turnover in the 'acid-soluble' phosphorylated intermediates of frog sciatic nerves was inhibited during stimulation, while the present report has been extended to include most of the structural components ('acid-insoluble') containing phosphorus. Since most phosphorylative activity in the nervous system is coupled to adenine nucleotides, it is not surprising that the phosphate turnover of lipids and nucleic acids is decreased, in view of the observations on the inhibition of ATP and phosphocreatine turnover.

As has been discussed previously (ABOOD and GOLDMAN, 1956) electrical excitation of neural tissue involves a physiological 'uncoupling' of phosphorylation and oxidation, in so far as phosphorylation is diminished while respiratory processes are greatly accelerated. It would appear that during excitatory processes in nerve, as well as muscle, energy utilization or catabolic reactions predominate over endergonic reactions. It is conceivable that the splitting and formation of 'high energy' compounds, such as ATP, are carried on by the same enzymatic complexes (e.g. mitochondrial) and that if degradative reactions predominate, synthetic ones are accordingly inhibited. The significance of the 'uncoupling' effect from the point of view of neural function is obscure. It has been speculated that in order for sodium to enter, a transitory inhibition in the 'sodium pump' mechanism may occur during depolarization (GRUNDFEST, 1950), and if such a mechanism is involved, it would be expected that a corresponding decrease should occur in phosphorylation, which is the chief energetic pathway.

It has been proposed that excitatory processes in nerve are accompanied by changes in the structural configurations (ABOOD and GOLDMAN, 1956; ABOOD and ABUL-HAJ, 1956), and that the role of ATP may be involved in the maintenance of such structures. Recently, ATP was presumed to play the role of catalyst rather than an energy donor in cellular metabolism (HART, 1957). By forming an activated complex stabilized through chelation or induction, it may be catalysing many different types of reactions that are otherwise slow. Among such reactions may be those involved in actual protein rearrangement and synthesis (LIPMANN, 1954).

Among the numerous substances examined, the free amino acids showed the most marked and consistent decrease during stimulation of frog nerves; this observation confirms the finding of MULLINS (1953) that amino acid utilization is accelerated during excitation of frog nerves, while carbohydrate metabolism is diminished. Evidence in support of the hypothesis that non-carbohydrate metabolism is accelerated during activity in neural tissue continues to accumulate (GREIGER *et al.*, 1956).

Numerous investigators (ABOOD *et al.*, 1952; DELUCA *et al.*, 1953; DAWSON, 1954) have noted the high rate of ^{32}P turnover of inositol metadiphosphate in neural tissue, but its significance is completely obscure. The fact that the turnover rate of inositol phosphate is inhibited during excitation of frog nerve implicates a relation to ATP turnover which is likewise inhibited. It remains to be determined whether the 'inositol phosphate' described in the present experiments originates from diphosphoinositide.

Although the total NA content of mammalian peripheral nerve was not found to change during stimulation, the RNA itself increased while the DNA decreased (CAUSEY and STRATMANN, 1956*a*). Preganglionic excitation of the superior cervical ganglion, however, leads to the reverse situation with essentially no change in total NA (CAUSEY and STRATMANN, 1956*b*). As much as a 300 per cent increase in the DNA

of the ganglion was reported to have occurred after 3 hr of stimulation. In view of the many substances other than NA components extracted with hot trichloroacetic acid responsible for ultraviolet absorption, the NA determinations of CAUSEY and STRATMANN (1956*a*, *b*) are open to question. The authors determined NA and DNA by their optical absorption at 268 mμ, a wavelength at which substances such as polypeptides, tyrosine, and tryptophane also absorb to a considerable extent. The advantage of ion exchange chromatography is that it permits the separation of the NA nucleotides from the many interfering substances.

The relatively slight changes in the total quantity of structural components of peripheral nerve as a result of stimulation are, perhaps, somewhat surprising, in view of the dramatic changes reported to take place in the central nervous system. A number of reports have appeared on the changes in nucleoprotein content within the nerve cell body (HYDÉN, 1943, 1951), and the whole brain (GEIGER *et al.*, 1956) following stimulation. Whether the difference is attributable to the fact that the metabolic rate of the nerve fibre is but a fraction of that of the cell body, or that an actual qualitative difference prevails in the metabolism associated with activity cannot be determined on the basis of the present findings. Yet, on the other hand, it can be argued that the major chemical changes associated with activity in the nerve fibre are not 'degradative' or catabolic, but rather involve intramolecular or structural rearrangements of components important in ion transfer (ABOOD and ABUL-HAJ, 1956). The observations of UNGAR *et al.* (1957) that excitation involves reversible changes in the configuration of nerve proteins is particularly noteworthy in this respect. Future work in our laboratory is directed along the lines of investigating such changes in the structural components of neural tissue.

SUMMARY

The effect of electrical stimulation on phosphorus turnover of the nucleic acid and phospholipid components of bullfrog sciatic nerves has been investigated, using ^{32}P-orthophosphate. No significant decrease was observed in any of the nucleotide components of nucleic acid, although a slight decrease occurred in the phosphate turnover of RNA. A significant inhibition occurred in the turnover of the phospholipids and in a phosphatide-peptide complex as a result of excitation. The significance of the results is discussed in terms of the role of phosphorylation in conduction.

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THE UPTAKE AND OXIDATION OF GLUCOSE BY THE PERFUSED CAT BRAIN*

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(*Received* 23 *September* 1957)

VOL.
2
1957/5

THERE is general agreement that under normal physiological conditions, glucose is the main substrate for the oxidative metabolism of the mammalian brain. This viewpoint is supported by numerous *in vivo* studies on animals and humans as well as by *in vitro* studies, and it is stressed in many current discussions of the metabolism of the brain. On the other hand there is experimental evidence, derived from brain perfusion experiments carried out in this laboratory, that brain functions and oxidative metabolism may continue for short periods of time in the absence of glucose (GEIGER, MAGNES and GEIGER, 1952), and that in brain perfusion experiments carried out with semi-synthetic 'simplified blood' there may be significant alterations in the metabolism of glucose by the brain (GEIGER, MAGNES, TAYLOR and VERALLI, 1954).

In previous experiments it was found that when the brain of the cat is perfused for some minutes with a 'simplified blood,' the glucose content of the brain tissue decreases to very low values (10–30 mg/100 g) and that its lactate content increases considerably (GEIGER *et al.*, 1954). It was also shown that perfusion of the brain with an isolated liver included in the circulation restores the glucose and lactate levels to normal. A suggested interpretation of this finding was that in the absence of certain substances originating from the liver, the brain cells became impermeable to glucose.

The present experiments demonstrate that, in spite of the low glucose content of the brain, which results from perfusion with simplified blood, glucose uptake by the brain continues at a rapid rate. Furthermore, experiments with radioactive glucose, performed in order to determine to what extent the glucose taken up was serving as the substrate for oxidative processes, showed that the major part of the CO_2 produced by the brain was not derived from the glucose taken up.

METHODS

Isolation of the cerebral circulation was carried out according to the method of GEIGER and MAGNES (1947). *The perfusion apparatus* (Fig. 1) was modified to meet the following requirements:

(a) to obtain a constant flow of blood to the brain in order to stabilize A–V differences and facilitate the calculation of metabolic exchanges between blood and brain;

(b) to avoid the haemolysis of the red blood cells which results from the use of roller or finger pumps;

(c) to minimize metabolism of the red blood cells; and

(d) to provide a closed system which would allow the use of radioactive materials.

* Preliminary report presented at the International Congress of Physiology, Brussels, 1956 (ALLWEIS and MAGNES, 1956).

† Part of the data in this paper was taken from a thesis submitted by C. ALLWEIS to the Senate of the Hebrew University, in partial fulfilment of requirements for the degree of Doctor of Philosophy.

These requirements were met as follows:

The blood was displaced from a closed bottle by means of water under constant pressure (about 800 mm Hg) entering the rubber balloon (*E*) inside the bottle. The water flow was controlled manually by the needle valve (*B*) and measured with the rotameter (*C*). Changes in cerebral vascular resistance were recorded by means of a mercury manometer (*H*).

Metabolism of glucose by the red blood cells was minimized by immersing the blood reservoir in ice and rapidly heating the blood up to 37°C, with the silver heating coil (*J*), just before its entrance into the carotids. The venous blood, which was cooled down immediately upon emerging from the brain by a similar cooling coil (*M*), was collected under paraffin oil in NaF-coated cylinders immersed in the ice bath (*Q*).

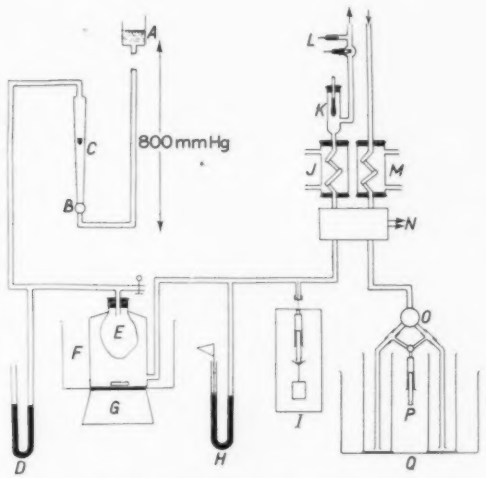

FIG. 1. Schematic diagram of perfusion apparatus.
A, Water reservoir; *B*, Needle valve; *C*, Rotameter; *D*, Safety manometer; *E*, Rubber balloon; *F*, Ice bath; *G*, Magnetic stirrer; *H*, Recording manometer; *I*, Constant rate injection machine; *J*, Heating coil; *K*, Thermometer pocket; *L*, Electrodes for pH measurement; *M*, Cooling coil; *N*, A–V Oximeter; *O*, 2-way stopcock; *P*, Paraffin-filled syringe; *Q*, Ice bath with venous collecting cylinders.

The venous samples were usually collected during 6 min periods. Venous blood collected during the first few minutes of perfusion was discarded to ensure thorough washing out of the dead space in the venous side of the apparatus. The arterial blood was sampled anaerobically at the beginning and end of each experiment. The virtually constant composition of the arterial blood in these experiments, together with the constant flow and the collection of venous samples over relatively long periods, provided favourable conditions for the accurate determination of metabolic exchanges between blood and brain, as compared with conditions obtaining in experiments in which the same blood was continually recirculated.

Respiration was recorded from a *T*-piece in the tracheal cannula, and peripheral arterial pressure from a femoral artery. A–V O_2 differences were recorded with the oximeter described by GEIGER and MAGNES (1947) and electro-cortical activity was recorded from various points on the surface of the cortex through burr holes in the skull. In all the experiments cerebral blood flow was set at 30 ml/min which was equivalent to 110–140 ml/100 g brain/min, depending on the weight of the brain which was determined at the end of each experiment.

Preparation of the simplified blood for perfusion. This was as follows:

The red blood cells of defibrinated bovine blood were washed exhaustively in Krebs-Ringer bicarbonate solution (UMBREIT *et al.*, 1949) and finally suspended in Krebs-Ringer bicarbonate solution containing 7 per cent bovine albumin, to give an haematocrit of 36–38 per cent. The

albumin was previously dialysed in the cold to remove vasoconstrictor substances, and its pH adjusted to 7·4 at 37° by addition of NaOH. By careful attention to the CO_2 content of the Krebs-Ringer bicarbonate solution, and by keeping all containers hermetically sealed throughout the preparation, the final pH of the blood was always very close to 7·4 at 37°. Glucose (100 mg) was added to each 100 ml of blood shortly before starting perfusion. For experiments with radioactive glucose, uniformly ^{14}C-labelled glucose was diluted with carrier glucose to give a final activity of 1–2 μC/g.

Analytical methods. Serum glucose determinations* were made in duplicate by the SOMOGYI (1952) iodometric method, following precipitation with $Ba(OH)_2$ and $ZnSO_4$. Brain glucose and lactate were determined in tissue samples weighing 0·1–0·2 g. Before stopping the perfusion, part of the cortex was exposed, and a thin slice of tissue was rapidly excised with a scalpel and transferred to a thin sheet of aluminium resting on CO_2 ice. The tissue sample was thus completely frozen within 30 sec after being removed from the animal. An extract was prepared with $Ba(OH)_2$ and $ZnSO_4$ and analysed for glucose using NELSON'S (1944) colorimetric modification. Lactate was determined by the method of BARKER and SUMMERSON (1941).

Radioactivity analyses and calculations. The liberation of CO_2 from the blood samples and absorption in NaOH were carried out with the simple apparatus shown in Fig. 2. Intravenous infusion bottles were used. Before the experiment two 25 ml test tubes, each containing about 5 ml of 1 N-NaOH (CO_2-free), were placed in the bottles which were immediately closed. The bottles were then evacuated down to a pressure of about 20 mm Hg through a hypodermic needle piercing the 'vaccine type' cap at the upper end of the central tube. About 200 ml of 0·2 N-lactic acid (CO_2-free) and a few drops of capryl alcohol were then introduced through the cap. As each venous blood sample was collected from the cat, 40–60 ml were transferred into a prepared bottle through a thin polyethylene tube ending in a hypodermic needle. Arterial samples were treated similarly. After mixing, the bottles were allowed to stand with occasional shaking for 4–6 hr at room temp., and then opened after releasing the vacuum with a needle. Quantitative recovery of the blood CO_2 was not necessary. The duplicate tubes containing the absorbed CO_2 were withdrawn and about 5 ml of 1 N-$BaCO_3$ added to each. The precipitated $BaCO_3$ was washed and plated with precautions to avoid contamination with room CO_2. The $BaCO_3$ plates were above saturation thickness and all counting was done with a Geiger-Müller mica end-window tube.

In addition a standard $BaCO_3$ plate was prepared from the radioactive glucose solution used in each experiment, by chemical oxidation *in vitro* (CALVIN, HEIDELBERGER, REID, TALBOT and YANKWICH, 1949). Arterial and venous blood samples, for Van Slyke analyses for CO_2 and O_2 content, were stored in syringes in an ice bath and analysed on the same day.

Since the arterial blood contained about 40 vol per cent of inactive CO_2, the radioactive CO_2 derived from the brain underwent dilution. In order to correct for this dilution, the final activities of the $BaCO_3$ plates were multiplied by their respective dilution factors which were derived from the Van Slyke analyses of the arterial and venous samples.

$$\text{Dilution factor} = \frac{\text{CO}_2 \text{ vol \% in venous blood}}{\text{CO}_2 \text{ vol \% in venous blood} - \text{CO}_2 \text{ vol \% in arterial blood}}$$

Since all activities were determined at saturation thickness, the activity of the $BaCO_3$ derived from the venous CO_2 multiplied by the appropriate dilution factor was immediately comparable with the activity of the $BaCO_3$ derived by direct chemical oxidation of the ^{14}C-glucose used in the experiment.

$$\text{Thus} \quad \frac{\text{c/min } BaCO_3 \text{ from venous blood} \times \text{dilution factor}}{\text{c/min } BaCO_3 \text{ from chemical oxidation of radioactive glucose}} \times 100 = \% \, CO_2$$

produced by brain which was derived from radioactive glucose in the blood.

* *Serum versus 'whole' blood glucose.* The glucose content of 'whole' simplified blood and the glucose content of the corresponding 'serum' obtained by centrifuging down the bovine red cells were determined for both arterial and venous samples in many experiments. It was found that 'whole' blood glucose values were always very close to 70 per cent of the corresponding serum values, with haematocrit values of 36–38 per cent. Allowing for the small amount of serum included in the centrifuged red cells, these results indicate that about 93 per cent of the glucose in simplified blood is present in the serum. Consequently all of the A–V analyses for the calculation of glucose uptake were carried out on serum in order to avoid errors due to varying proportions of red cells and serum in blood samples.

The use of radioactive glucose in the perfusing blood furthermore makes possible an accurate calculation of the rate of oxidation to CO_2 of glucose taken up from the blood by the perfused brain, irrespective of the oxidation of other substrates which may be occurring simultaneously.

The only data required for this calculation are:

(1) The CO_2 content of the venous blood in vol% = CO_2 final.

(2) The activity (c/min at saturation thickness) of $BaCO_3$ derived from the CO_2 in the venous blood = c/min final.

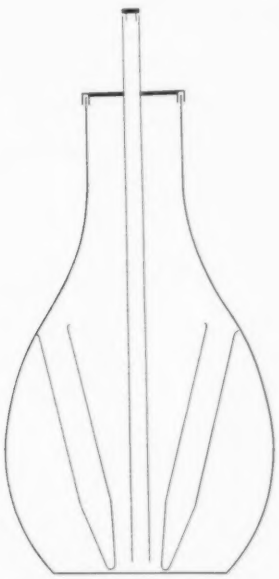

FIG. 2. Apparatus for the transfer of CO_2 from blood to CO_2-free NaOH (details in text).

(3) The activity (c/min at saturation thickness) of $BaCO_3$ derived directly from the radioactive glucose used in the experiment by chemical oxidation = c/min radioactive glucose.

It is convenient to consider 100 ml of blood containing radioactive glucose which is passed through the brain. The relevant changes in its composition which occur are:

(a) The oxidation of a certain amount of glucose (m mg) to CO_2.

(b) The addition of a corresponding amount of CO_2 (i.e. $0{\cdot}747 \times m$ ml) to the CO_2 already present in the arterial blood.

(c) The introduction of a certain amount of CO_2 derived from the oxidation of substrates other than glucose in the blood.

Thus the dilution of the ^{14}C-CO_2 derived from the oxidation of radioactive glucose, by the CO_2 present in the arterial blood and the CO_2 in the venous blood derived from the oxidation of substrates other than glucose, will be equal to

$$\frac{CO_2 \text{ final}}{m \times 0{\cdot}747}$$

Now the ratio c/min ^{14}C-glucose:c/min final is similarly a measure of the dilution of CO_2 derived from the oxidation of radioactive glucose, i.e.

$$\frac{CO_2 \text{ final}}{m \times 0{\cdot}747} = \frac{\text{c/min } ^{14}\text{C-glucose}}{\text{c/min final}}$$

therefore $$m = \frac{CO_2 \text{ final} \times \text{c/min final}}{0{\cdot}747 \times \text{c/min } ^{14}\text{C-glucose}}$$

and the rate of ^{14}C-glucose oxidation expressed as mg/100 g brain/min

$$= \frac{m}{100} \times \text{blood flow in ml/100 g brain/min}$$

This method of calculation does not require the measurement of A–V differences and is thus free from the inaccuracies inherent in such measurements.

RESULTS

Glucose uptake. This series of experiments was carried out in order to determine to what extent the perfused brain was capable of taking up glucose from 'simplified blood.' Brain function, as estimated by the corneal reflex, EEG, and respiration was usually good at the start of perfusion, but tended to deteriorate as the experiment

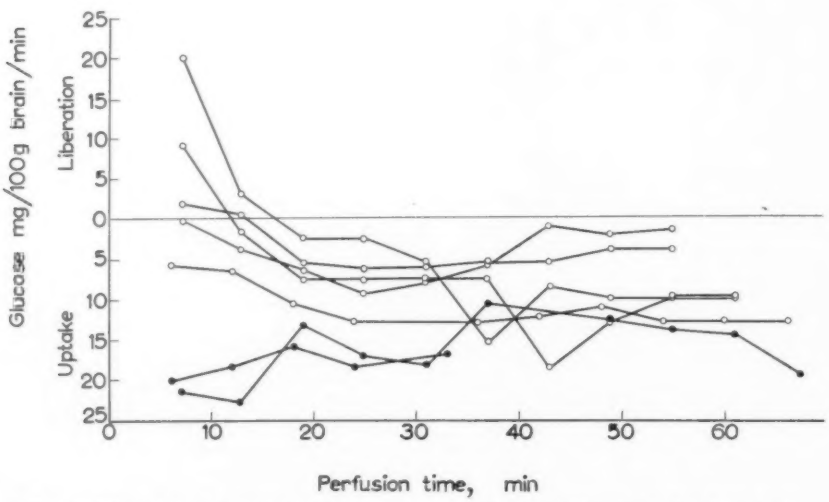

FIG. 3. Liberation and uptake of glucose from simplified blood by the perfused cat brain. ○—○—○ = controls; ●—●—● = cats received insulin about 3 hr before perfusion.

progressed. The arterial serum glucose concentration was between 110 and 150 mg/100 g. Glucose uptake was determined by A–V differences over 6 min periods throughout the experiment as described above. The results are presented in Fig. 3 and are expressed as mg glucose taken up, or liberated, per 100 g brain/min.

Table 1 gives mean oxygen and glucose uptakes over selected periods of from 30 to 59 min. These periods were selected from five experiments, as being prolonged periods during which both oxygen utilization and glucose uptake were fairly constant. Theoretical glucose uptakes, equivalent to the measured oxygen utilization, are included, and the last column represents the glucose concentration in the brain at the end of the experiment.

From the data presented it may be seen that during the first 10 min or so of perfusion the brain often liberated reducing substances into the blood. Subsequently

the brain began to take up glucose from the blood at a rate which finally became fairly constant at an average of 9·1 mg/100 g brain/min. The corresponding average

TABLE 1.—GLUCOSE UPTAKE, OXYGEN UPTAKE AND TERMINAL BRAIN GLUCOSE CONCENTRATION IN BRAIN PERFUSION EXPERIMENTS

Date (1955)	Duration of exptl. period considered (min)	Glucose uptake (mg/100 g brain/min)	O_2 uptake (ml/100 g brain/min)	Glucose uptake theoret. calcul. from O_2 uptake (mg/100 g brain/min)	Reducing substances in brain at end of expt. expressed as glucose (mg/100 g)
17/5	48	10·1	6·6	8·8	25
7/6	30	10·8	9·1	12·2	—
14/6	42	5·2	5·7	7·5	21·4
5/7	54	12·4	4·7	6·3	20·5
16/8	24	7·3	8·5	11·3	13
Mean		9·16	6·9	9·22	—

rate of oxygen utilization was 6·9 ml/100 g brain/min. Thus, on the average, the glucose uptake was almost identical with the theoretical uptake calculated from the rate of utilization of oxygen, if it be assumed that all of the oxygen taken up was being used to oxidise glucose to CO_2 and water. The glucose content of the brain at the end of these experiments was invariably low, between 13 and 25 mg/100 g.

An explanation for the initial release of reducing substances into the blood in these experiments was sought in the difference in the serum glucose concentration in the cat's blood and that of the perfusing blood. It was found in several experiments that the serum glucose concentration in the cat's blood rose during the period of the operation and had attained values as high as 300 mg/100 g by the time perfusion was begun. It was considered that this high serum glucose level might result in a high glucose concentration in the brain, and that part of this glucose might be released into the blood when perfusion was commenced with a much lower level of serum glucose.

To clarify this point, two perfusion experiments were performed on cats which had received insulin at the commencement of the operation and whose serum glucose values at the start of perfusion about 3 hr later were consequently very low (about 20 mg/100 g). The results of these experiments are included in Fig. 3. In neither case was there a release of glucose into the blood. Both showed a high initial rate of glucose uptake (about 20 mg/100 g brain/min) which diminished during the course of the perfusion, but remained higher than the rate of uptake usually found in cats that had not received insulin.

These experiments, although they confirm previous findings of a low concentration of glucose in the brain after about 1 hr of perfusion with simplified blood, indicate that this phenomenon is not due to impermeability of the brain to glucose.

The fact that the brain was able to take up glucose at a rate adequate to cover the rate of utilization of oxygen does not necessarily mean that the glucose taken up was the only substrate for its oxidative metabolism. In order to determine to what extent the glucose taken up was serving as the substrate for oxidative processes, further experiments were carried out with radioactive glucose.

The uptake and oxidation of radioactive glucose. The results of a number of experiments are presented in Fig. 4 which shows that the percentage of the CO_2

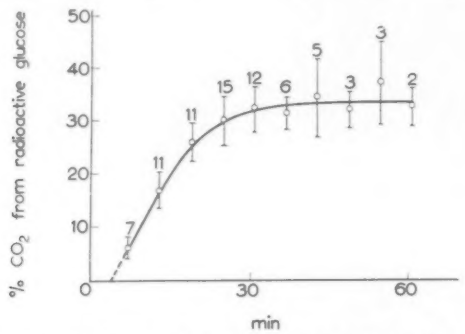

FIG. 4. The production of radioactive CO_2 by cat brain perfused with simplified blood containing uniformly labelled glucose. The vertical lines indicate standard deviations. The figure above each vertical line indicates the number of experiments from which the values were derived.

produced by the brain, which was derived from the radioactive glucose present in the arterial blood, rose within 30 min to a value of about 33 per cent and remained at this level for at least 35 min.

Extrapolation of this curve down to zero radioactivity indicates an apparent lag of about 4 min before any radioactive CO_2 is produced. This is in agreement with results shown in Fig. 3 which demonstrate liberation of reducing substances, probably glucose, from the brain into the blood during the first few minutes of perfusion, due to the change-over from cat blood with a very high concentration of glucose, to simplified blood with a much lower concentration of glucose.

Other data from twelve experiments are presented in Table 2. In the first six experiments the experimental periods selected were periods beginning after about 25 min of perfusion, during which the percentage of the venous CO_2 derived from radioactive glucose was fairly constant as indicated by the analysis of 6 min samples. The shortest period considered for calculation was 24 min. In the case of the remaining six experiments all of the venous blood was bulked for analysis and the data therefore apply to the entire perfusion period, which was usually about 60 min. Both series of experiments were therefore averaged for the complete series of twelve experiments.*

It may be seen that the mean rate of oxidation of radioactive glucose to CO_2 (calculated as indicated under METHODS) was 1·94 mg per 100 g brain/min. The mean rate of glucose uptake was 8·98 mg/100 g brain/min, and lactic acid was found to be liberated into the blood, at a mean rate of 4·9 mg/ 100 g brain/min. The mean rate of oxygen uptake was 7·52 ml per 100 g brain/min. The mean lactic acid content

* Expected differences between the results of these two series of experiments, owing to the inclusion of the equilibration period in the results of the second series, were probably within the experimental error.

of the brain cortex at the end of these experiments (determined on samples rapidly excised and frozen in solid CO_2) was 177 mg/100g. Taking the brain lactic acid content at the beginning of perfusion as 90 mg/100 g (GEIGER *et al.*, 1954), the mean rate of accumulation of lactic acid in the brain would be 1·5 mg/100 g brain/min. In a series of seven experiments, arterial and venous blood was sampled after 25 and

TABLE 2.—OXIDATION OF BLOOD GLUCOSE TO CO_2, GLUCOSE UPTAKE, LACTATE LIBERATION, AND OXYGEN UPTAKE OF CAT BRAIN PERFUSED WITH SIMPLIFIED BLOOD CONTAINING RADIOACTIVE GLUCOSE
(Figures given are mg or ml per 100 g brain/min)

Year	Date	Glucose uptake	^{14}C-Glucose to ^{14}C-CO_2	Lactate liberation	O_2 uptake
1955	2/8	12	1·43	3·3	—
	16/8	7·5	2·35	7·6	8·0
	8/11	—	2·54	—	9·5
	29/11	—	1·64	3·8	6·0
1956	17/1	7·0	1·28	3·3	5·5
	7/2	—	2·44	—	10·0
Mean		8·8	1·95	4·5	7·8
1956	19/5	6·0	1·49	2·3	8·0
	29/5	10·1	1·05	2·6	—
	5/6	6·0	1·90	4·5	4·5
	31/7	9·7	1—	—	7·2
	14/8	11·0	·69	7·7	6·5
	21/8	11·5	3·49	9·2	10·0
Mean		9·05	1·92	5·3	7·2
Mean for 12 experiments		8·98	1·94	4·9	7·52

31 min of perfusion, and the R.Q. of the metabolizing brain was determined by Van Slyke analyses. There was no significant difference between the R.Q. after 25 min and that after 31 min perfusion. The mean of all the R.Q. determinations performed was 0·79 with an S.D. of 0·145.

These experiments provide further evidence that the perfused brain is capable of taking up glucose from simplified blood, and oxidizing it to CO_2, but they indicate that only about one-quarter of the glucose taken up from the blood was oxidized to CO_2. The constant liberation of lactic acid from the brain and the high lactic acid concentration found in the brain at the conclusion of the experiments, suggested that a major part of the glucose taken up by the brain was subsequently converted to lactic acid, most of which was liberated into the venous blood. Only about 20 per cent of the oxygen taken up by the brain was used for the oxidation of blood glucose. Of the glucose taken up by the brain 95 per cent could be accounted for as CO_2 and lactate.

Oxidation of radioactive fructose. It has been shown that though brain slices respire at the same rate with either glucose or fructose (LOEBEL, 1925), this sugar is incapable of maintaining brain functions *in vivo* (MADDOCK, HAWKINS and HOLMES, 1939), possibly due to its slow rate of transfer from blood to brain (KLEIN, HURWITZ and OLSEN, 1946). When fructose was forced into the brain by the administration to the animal of massive doses, it did not disappear from the brain during brain perfusion experiments (GEIGER *et al.*, 1954) and the metabolic behaviour of the brain was similar to that found in perfusion experiments without glucose in the blood (GEIGER *et al.*, 1952). In view of these findings it was considered of interest to examine the oxidation of radioactive fructose in brain perfusion experiments.

Two experiments were performed in which the simplified blood contained 100 mg/100 g of uniformly labelled radioactive fructose. The procedures were as outlined in the experiments with radioactive glucose. Cerebral functional activity and O_2 consumption decreased rapidly in these experiments. The radioactivity of the venous CO_2 indicated that not more than 5 per cent of the CO_2 produced by the brain was derived from fructose. These experiments furnish direct evidence that the perfused cat brain is able to utilize fructose as a substrate for oxidation only to a very limited extent.

DISCUSSION

The results described above confirm the previous finding of GEIGER *et al.* (1954) that the concentration of glucose in the cat brain after perfusion for one hour with simplified blood is very low (13–25 mg/100 g). Their suggestion that this low brain glucose concentration is due to an impairment of the permeability of the brain to glucose, would appear to be untenable, since in all the experiments here described (Table 1) glucose uptake was demonstrated by measurements of A–V differences. Although the glucose uptake (9·1 mg/100 g brain/min) was adequate to provide the substrate for the oxygen utilized (6·9 ml/100 g brain/min), later experiments showed that the brain was also producing considerable quantities of lactic acid which was accumulating in the brain at a rate of approximately 1·5 mg/100 g brain/min and being liberated into the blood at a rate of approximately 5·3 mg/100 g brain/min. Thus, if the lactic acid produced by the brain was derived from blood glucose, then only about 22 per cent of the glucose entering the brain would be available for oxidation to CO_2, the rest being converted to lactic acid. Such a situation would imply that only about 20 per cent of the oxygen taken up by the brain was being used to oxidize exogenous glucose to CO_2, whereas the remainder was being used to oxidize other endogenous substrates.

In subsequent perfusion experiments with radioactive glucose (Table 2) this supposition was confirmed. It was found that the mean rate of oxidation of radioactive glucose to CO_2, calculated as described earlier, was 1·9 mg/100 g brain/min when the total glucose uptake was 9·0 mg/100 g brain/min, and the radioactivity of the CO_2 produced by the brain had reached an approximately constant value after about 30 min of perfusion. Thus, in these experiments, only 22 per cent of the glucose taken up from the blood was oxidized to CO_2. Most of the remaining glucose taken up could be accounted for as lactate (6·4 mg lactic acid/100 g brain/min) accumulated in the brain and liberated into the blood, but 7 per cent of the glucose taken up could not be accounted for. The high rate of aerobic glycolysis during perfusion of the cat brain with simplified blood has been noted previously (GEIGER *et al.*, 1954).

The finding that the fraction of the total CO_2, produced by the brain during perfusion, which is derived from the radioactive glucose in the blood, rises within 30 min to a value of approximately 33 per cent and then remains almost constant at this value for at least another 35 min, provides further evidence for the preferential oxidation of endogenous substrates.

The unlabelled glucose and glycogen initially present in the brain must be considered as possible sources of the lactate and unlabelled CO_2 produced by the brain. Their initial concentrations may be estimated at 100 mg/100 g each, (KLEIN and OLSEN, 1947) equivalent to a total of 80 mg/100 g of C.

The total amount of carbon liberated by the brain during the 60 min perfusion period was equal to approximately 165 mg as lactate plus 160 mg as CO_2 (calculated from an average rate of CO_2 production of 5·1 ml/100 g brain/min as determined by Van Slyke analyses) i.e. a total of 325 mg. It may be assumed that blood glucose, brain glucose and glycogen are all metabolized in the same manner, via pyruvate to CO_2 and lactate. Thus, if all the brain glucose and glycogen were completely metabolized to CO_2 and lactate within the 60 min experimental period, they would account for about 25 per cent of the CO_2 and 25 per cent of the lactate produced.

It remains to be seen whether the carbohydrate stores are in fact responsible to this extent for the low radioactivity of the CO_2 produced by the brain, since brain glycogen values during perfusion are not yet available. It is none the less clear that the major part of the unlabelled CO_2 produced by the brain must have been derived from other endogenous non-carbohydrate substrates, and further evidence for this may be derived from the R.Q. of 0·79.

The relationship between the metabolism of the cat brain perfused with simplified blood and its metabolism in the intact animal is not clear. GEIGER and YAMASAKI (1956) have recently reported that the addition at intervals of about 2·5 mg/100 g of uridine and cytidine to simplified blood maintains the perfused brain in good functional condition with a normal carbohydrate metabolism for 4 hr or longer.

We have carried out two perfusion experiments with radioactive glucose—using simplified blood containing 2 mg/100 g each of uridine and cytidine. The fraction of the CO_2 produced by the brain which was derived from radioactive glucose was the same as that found in the controls without uridine and cytidine.

The experiments with radioactive fructose demonstrate directly that the perfused brain is able to oxidize fructose to CO_2 only at a very limited rate. With the blood concentration used (100 mg/100 g) this result could well be due to the limited rate of penetration of fructose into the brain.

SUMMARY

Glucose uptake and oxidation by the cat brain were studied in perfusion experiments. About one-quarter of the glucose taken up is oxidized to CO_2 accounting for approximately one-fifth of the O_2 consumed. Most of the remaining glucose is converted to lactic acid. Using radioactive glucose, evidence was obtained suggesting that the greater part of the remaining 80 per cent of the O_2 was used for the oxidation to CO_2 of non-glucose endogenous substrates within the brain.

Acknowledgements—We are indebted to Professor B. SHAPIRO of the Dept. of Biochemistry for helpful discussion. These experiments owe much to the technical assistance of D. BEN-SHAHAR,

SH. BEN-YONAH, L. KATZ and L. TALVI. A grant from the Hadassah Medical Organization Research Fund made possible the purchase of radioactive glucose.

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CHANGES OF BRAIN GLYCOGEN IN THE SPREADING EEG—DEPRESSION OF LEAO

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(*Received* 24 *October* 1957)

THE spreading depression of EEG activity is one of the manifestations of cerebral cortical functions which has not, so far, been chemically analysed. Various electrical, mechanical and chemical stimuli evoke at their point of action on the surface of the cerebral cortex a local decrease in the amplitude of the EEG, which spreads slowly at a rate of 3–6 mm/min along the surface of the hemisphere (LEAO, 1944). The front of the depression wave is accompanied by a negative potential lasting for 1 min and reaching an amplitude of 7–10 mv. The spreading depression (sp. D.) is independent of the depth of narcosis or of short-lasting anoxia (LEAO and MORISON, 1945; LEAO, 1947). According to some authors (SLOAN and JASPER, 1950; MARSHALL, ESSIG and DUBROFF, 1951) nerve contiguity is the chief requirement for the spreading.

These findings suggest our seeking the basic mechanism of the EEG depression in humoral transmission and in the metabolic processes which underlie it. Some evidence in support of this is given by the work of BUREŠ (1956), in which it was shown that sp. D. is evoked by substances such as L-glutamine, NH_4Cl, KCl, 2:4-dinitrophenol and veratrin, which cause increased respiration and anaerobic glycolysis of brain slices *in vitro* (ASHFORD and DIXON, 1935; WEIL-MALHERBE, 1938; GHOSH and QUASTEL, 1954; WOLLENBERGER, 1955), if they are applied locally to the surface of the brain. Conversely substances that prevent the effects of increased potassium ion concentration on the respiration and glycolysis of brain slices, e.g. citrate, α-ketoglutarate, succinate and malonate (LIPSETT and CRESCITELLI, 1950; MCILWAIN, 1954; KIMURA and NIWA, 1953), prevent the onset of the sp. D. evoked by KCl, when added in suitable concentrations to the KCl solutions applied to the brain surface. The work of VAN HARREVELD and STAMM (1954), who found a decrease in the oxygen tension in the cerebral cortex during sp. D., gives further evidence in support of the view that there is a connection between the onset of sp. D. and the effects of the above substances on the metabolism of brain slices *in vitro*. This decrease is not caused by vasoconstriction, as was originally thought by these authors, since BUREŠOVÁ (1957) has shown, by haemoglobin determinations, by measurements of the uptake of ^{24}Na, ^{32}P and ^{42}K into cortical regions of the control and experimental hemisphere and by thermo-electric measurements, that the sp. D. is accompanied by a vasodilatation. The lowered oxygen tension during sp. D. is, therefore, caused by increased oxygen uptake by the brain tissue.

In the present report an attempt was made to find a relationship between the action of potassium ions *in vitro* and *in vivo* and changes in the carbohydrate metabolism during sp. D. The study was commenced by determining the glycogen content of the rat brain in the course of the sp. D. evoked either mechanically or by local application of KCl at various concentrations to the surface of the brain.

METHODS

Experiments were performed on rats of about 150 g weight, anaesthetized with dial (40 mg/kg by intraperitoneal injection). A trephine opening 5 mm in diameter was made above the occipital region of both hemispheres. Into the opening, a filter paper (2 × 2 mm) which was soaked in a 2% or 25% aqueous solution of KCl, was applied to the experimental hemisphere, while a filter paper soaked in saline solution was applied to the opening in the other control hemisphere. Electrophysiological data concerning the effect of the KCl solutions applied in this way can be found in previous papers from our laboratory (BUREŠ and BUREŠOVÁ, 1956). After the appropriate time interval following the application of the KCl solution the rats were decapitated and the heads dropped into liquid air. A brass rod of 8·7 g weight and about 8·55 mm² cross-section, which was dropped by its own weight onto the brain surface from a height 7·5 mm, was used to evoke sp. D. by mechanical means.

The glycogen content was determined in both hemispheres according to KERR (1936); the reducing compounds formed after acid hydrolysis of glycogen were determined by the method of NELSON (1944). The results are expressed in mg hexose per 100 g wet weight ± standard error of the mean (S.E.M.). The statistical evaluation was performed by Fischer's *t*–test.

RESULTS

The influence of sp. D. *evoked by* 2% KCl *on the glycogen content.* The sp. D. evoked by the application of 2% KCl solution was accompanied by a considerable decrease in the content of glycogen in the experimental hemisphere. Five minutes after the application of the filter paper soaked in KCl the difference was already statistically significant for $P < 0{\cdot}01$. The maximum decrease was reached 30 min after the application of the filter paper. After 60 min a partial return to normal was seen, but the lowering was still statistically significant ($P < 0{\cdot}01$). The glycogen content returned to normal 90 min after the application. The difference in the glycogen content between the two hemispheres was then no longer statistically significant and it corresponded only to the normal variation.

The influence of sp. D. *evoked by* 25% KCl *on the glycogen content.* The course of the decrease of glycogen evoked by 25% KCl did not differ in the beginning from that following the application of 2% KCl, but a difference was noted in the duration of the maximum decrease and in the time at which the return to normal took place. The maximum decrease of 29·7 per cent was reached 60 min after application of the KCl solution and it was still present (28·2 per cent) 60 min later. After this the glycogen content began to rise again and 210 min after application the decrease was 12·1 per cent, which was statistically significant for $P < 0{\cdot}01$. At 300 min after the application the glycogen content had returned to normal.

The influence of sp. D. *evoked by mechanical means on the glycogen content.* In order to eliminate the possibility that the changes in glycogen content were due to a specific effect of the KCl, the sp. D. was evoked by a basically different means. Making use of the results of ZACHAR and ZACHAROVÁ (1957), we found in preliminary experiments under EEG control that the impact of a brass rod 8·7 g in weight with a 8·55 mm² contact surface consistently evoked a wave of depression. Four minutes later it was already possible to evoke further waves. For determining the changes in glycogen content accompanying the sp. D. evoked in this way, three stimuli following each other at 4 min intervals were used; 10 min after the first stimulus the rats were decapitated and glycogen was determined.

As can be seen in Table 1, the sp. D. evoked by mechanical stimuli was accompanied by a change in the glycogen content similar to that in the case of the sp. D. evoked by

KCl. The mean decrease in the glycogen content was 23·8 per cent and it was statistically significant for $P < 0{\cdot}01$.

TABLE 1.—EFFECT OF THE SPREADING DEPRESSION OF EEG ACTIVITY ON THE LEVEL OF BRAIN GLYCOGEN IN THE RAT

sp. D. evoked by	Interval between application of agent and fixation of the tissue (min.)	Number of rats	Glycogen content (mg hexose/100 g brain)		Change (%)	Statistical significance (P)	
			Control hemisphere S.E.M.	Experimental hemisphere S.E.M.			
25% KCl	5	8	119·8 ± 3·5	107·8 ± 3·4	−10·0	12·0 ± 4·2	N.S.
	10	8	94·9 ± 11·8	78·8 ± 8·3	−17·0	16·1 ± 7·4	N.S.
	30	16	124·5 ± 4·6	90·2 ± 2·7	−27·6	34·3 ± 2·4	0·01
	60	7	109·5 ± 7·5	77·0 ± 6·7	−29·7	32·5 ± 5·9	0·01
	120	9	95·3 ± 3·8	68·5 ± 5·5	−28·2	26·8 ± 2·7	0·01
	210	9	103·0 ± 3·6	90·5 ± 4·9	−12·1	12·5 ± 3·0	0·01
	300	13	105·0 ± 3·8	108·7 ± 4·5	+3·5		
2% KCl	5	6	95·7 ± 4·8	83·2 ± 4·5	−13·0	12·5 ± 2·7	0·01
	10	15	96·3 ± 2·2	79·1 ± 3·0	−17·5	16·8 ± 2·0	0·01
	30	11	94·9 ± 3·1	68·2 ± 4·0	−28·1	26·7 ± 3·8	0·01
	60	10	102·3 ± 2·6	87·8 ± 2·3	−14·2	14·5 ± 2·6	0·01
	90	7	98·8 ± 4·1	93·9 ± 2·7	−5·0	4·9 ± 2·5	N.S.
Mechanically		11	93·5 ± 3·4	71·0 ± 3·9	−24·1	22·5 ± 3·5	0·01

DISCUSSION

It has been shown in our experiments that waves of sp. D. are accompanied by a considerable decrease in the glycogen content of the cerebral hemisphere concerned. We conclude that these results give further evidence that the action of potassium ions *in vivo* is analogous to their action on brain tissue slices *in vitro*. It is well known from *in vitro* experiments that an increase in the concentration of potassium ions in the medium to more than 40 mM leads to an inhibition of glycogen synthesis in brain slices (KLEINZELLER and RYBOVÁ, 1957). A similar effect was noted after the addition of 2:4-dinitrophenol or after electrical stimulation of the brain slices (LEBARON, 1955). MCILWAIN and TRESIZE (1956), were unable to observe the effect of electrical stimulation on the glycogen content of brain slices, but this can be explained by the low level of these substances in the brain slices.

The electrical manifestations of sp. D. disappear considerably earlier than the return of the glycogen level to normal. This was also found in other observations of the relations between electrical and chemical changes e.g. in anoxia (ALBAUM, NOELL and CHINN, 1953), and it may be related to the slow rate of glycogen synthesis in brain tissue *in vitro* (LEBARON, 1955) and *in vivo* (GAYEVSKAYA, 1953). A further analogy can be seen in the fact that an increased potassium ion concentration *in vitro* in the medium leads to increased respiration and an accumulation of lactic acid: the source of this *in vivo* may be glycogen as well as glucose (KERR and GHANTUS, 1937). It is evident that the action of potassium ions *in vivo* resembles

their action *in vitro* in their effect on the carbohydrate metabolism of brain tissue.

The mechanism of this effect of potassium ions is not yet clear. Although *in vitro* an increase in potassium ion concentration causes a rapid decrease in the phosphocreatine content and an accumulation of inorganic phosphate (HEALD, 1954), potassium ions do not appear to exert a specific influence on any of the enzymic reactions of the glycolytic or oxidative breakdown of carbohydrate in brain tissue. An effect similar to that of potassium has been demonstrated for a number of other substances such as glutamine, NH_4^+, 2:4-dinitrophenol, veratrine and aconitine, and for electrical and other stimuli. An increased concentration of potassium ions does not have the same effect on all tissues, and it is significant that the effect can be evoked only in systems with intact cells, i.e. not in tissue homogenates or extracts. It is therefore probable that the action of potassium ions, and of substances with a similar effect, is localized in the cell-membrane or it requires the integrity of the cell structure. It would appear that potassium ions cause exhaustion of energy stores *in vitro* as well as *in vivo*, either by increased demand or by retarded regeneration. The lowered electrical activity of the cortical neurons is an electrophysiological manifestation of this state.

Comparing our results with experiments in which the influence of various factors on the glycogen content of the brain have been studied, it can be seen that the sp. D. exerts a relatively large effect on the carbohydrate metabolism. Fasting for 4 days has little effect on the glycogen content of the dog brain (KERR and GHANTUS, 1936) or the mouse brain (CHANCE and WALKER, 1954). Hypoxia in dogs evoked by inhalation of a gas mixture deficient in oxygen was without effect on brain glycogen (GURDJIAN *et al.*, 1949; GURDJIAN and WEBSTER, 1947) and it caused no appreciable change in the brain glycogen even of dogs in metrazole convulsions, although there was an accumulation of lactic acid and a decrease in the phosphocreatine content. Narcosis was also without effect on the glycogen content of the mouse brain (CHANCE, 1953) and of the cat (OLSEN and KLEIN, 1947); PALLADIN (1954), however, reported a 40 per cent increase in the polysaccharides of the rabbit brain. A similar finding was reported by LEPAGE (1946). Brain glycogen changes only after a radical interference with the normal function of the brain. Longer fasting, for 6–27 days, leads to a drop in the glycogen content of the rabbit brain, and this is even more rapid than in the liver (KUMAMOTO, 1953). Severe anoxia (GAYEVSKAYA, 1953; ALBAUM *et al.*, 1953) causes a fall of 50–70 per cent in the brain glycogen. Similarly convulsions evoked by picrotoxin or by metrazole decrease the glycogen content of the cat brain by 40 per cent (KLEIN and OLSEN, 1947). Considerable changes in glycogen content, but in the opposite direction, were reported by CHANCE (1950, 1953, 1956) and his collaborators in the brains of mice following convulsions evoked by picrotoxin, metrazole or electrical stimulation, after paroxysms due to audiogenic epilepsy, after traumatic injury and after so-called 'offensive' behaviour. Despite the contradictory results, which may be due to differences in methods and in the animal species used, it is evident that the brain glycogen is a relatively stable metabolite, which is affected only by factors that exert a profound effect on the carbohydrate and phosphate metabolism.

The changes in glycogen accompanying the sp. D. are well marked. Experiments in which the sp. D. was evoked by mechanical means suggest that it was due, not to the specific action of KCl applied to the surface of the cerebral cortex, but rather

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to the resulting chemical changes in the brain. On the other hand, it is impossible to differentiate strictly between the action of K^+ ions and mechanical stimulation, when discussing the mechanism of origin and propagation of the sp. D. It can be assumed, by analogy with the changes occurring in brain slices following increased K^+ ion concentration, that stimuli evoking the sp. D. cause an increased accumulation of K^+ ions in the extra-cellular space, and that the K^+ ions are really responsible for the initiation and spread of the depression. The experiments of GRAFSTEIN (1956) who found that the wave of negative potential, which accompanies the sp. D. is

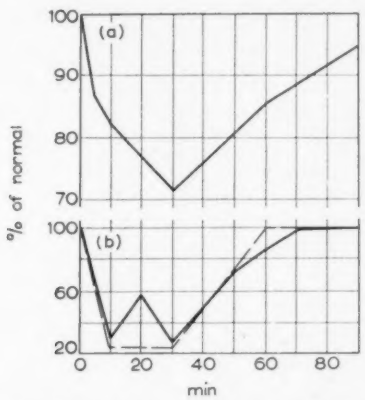

FIG. 1. Temporal correlation between changes in brain glycogen and the inhibition of conditioned reflexes (Burešová 1956*a*, *b*) both accompanying the spreading depression of EEG activity evoked by 2% KCl

(A) Glycogen content during sp. D. expressed as percentage of the glycogen content in the control hemisphere.

(B) Percentage of rats with correct responses in conditioned reflex experiments (– – – alimentary cond. reflexes; ——defence cond. reflexes).

preceded by a discharge lasting 2–3 sec, support this view. Furthermore she was able to influence the propagation of the sp. D. through a region of the cerebral cortex to which direct current was being applied, so that the wave of sp. D. advanced at a faster rate towards the negative than towards the positive pole. This experiment was interpreted by GRAFSTEIN as giving evidence that the sp. D. depends on the diffusion of a positively charged particle, and she concluded that the hyperactivity of the neurons preceding the sp. D. results in a liberation of potassium ions into the extra-cellular space. In this way the depolarization of neighbouring cells is achieved, and these in turn become stimulated and liberate more K^+ ions. It is evident, however, that as well as K^+ ions, nervous activity also plays an important role in the propagation of the sp. D., since it would not be possible to explain the rate of propagation by the diffusion of K^+ ions alone.

It is not yet possible to single out any one brain metabolite that can serve as an indicator of the functional state of the cerebral cortex. Attempts have been made to consider ammonia as indicator (VLADIMIROVA, 1954), but the functional significance of glycogen is not yet clear. It should be pointed out that there is a temporal relationship between the glycogen content of the rat brain and the duration of inhibition of the alimentary and defence conditioned reflexes studied by BUREŠOVÁ (1956*a*, *b*), who has shown that the sp. D. evoked by the topical application

of 2% KCl is accompanied by the inhibition of the reflexes studied. The maximum inhibition was reached between 10 and 30 min after the application of KCl solution, while the recovery to normal was attained about 60–70 min after the initial application of KCl (Fig. 1). With 25% KCl the maximum inhibition was still present after 60 min and complete restitution took 280–300 min (Fig. 2). On the basis of these

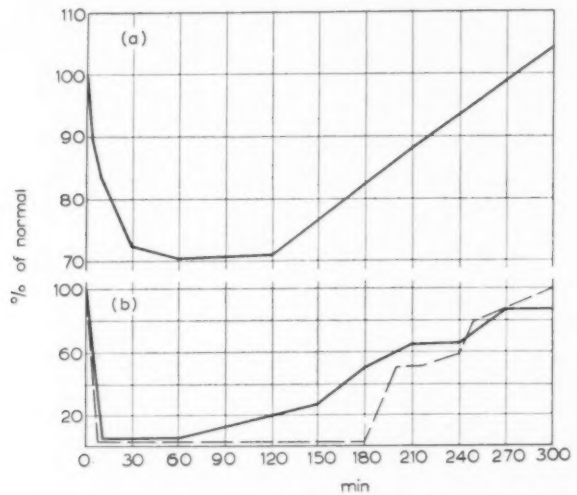

FIG. 2. Temporal correlation between changes in brain glycogen and the inhibition of conditioned reflexes (BUREŠOVÁ 1956*a*, *b*) both accompanying the spreading depression of EEG activity evoked by 25% KCl.

(A) Glycogen content during sp. D. expressed as percentage of the glycogen content in the control hemisphere.

(B) Percentage of rats with correct responses in conditioned reflex experiments (– – – alimentary cond. reflexes; ——defence cond. reflexes).

experiments BUREŠOVÁ concluded that the sp. D. is, physiologically speaking, a manifestation of deep cortical inhibition.

The reports cited indicate that the brain glycogen falls under conditions representing increased functional activity of the neurons. It could be assumed that the increased activity of individual neurons eliminates these cells from co-ordinated activity and so may lead to the inhibition of more complex neuronal mechanisms. Their restitution would not take place until the physiological activity of each cell was restored, making normal function again possible. The recovery of the glycogen level of the brain after various experimental stresses is known to occur later than that of the labile metabolites, such as lactic acid, glucose and phosphocreatine. It would therefore seem possible to consider the restitution of the normal glycogen level as an indication of the complete recovery of normal neuronal function in the brain.

SUMMARY

(1) Spreading depression (sp. D.) evoked by 2% KCl is accompanied by a statistically significant decrease of the glycogen content in the rat brain, with a maximum fall of 28·1 per cent reached 30 min after application of the KCl solution. At 90 min after application the glycogen level returned to normal.

(2) The sp. D. evoked by 25% KCl caused a similar decrease of the glycogen content, but the maximum fall (29·7 per cent) was maintained from 30 to 120 min after the KCl application. After 300 min the glycogen level returned to normal.

(3) The sp. D. evoked by mechanical stimuli applied at 4 min intervals also caused a statistically significant decrease of the brain glycogen content.

(4) Some metabolic and functional aspects of the sp. D. are discussed.

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